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CHEMICAL CHANGES IN THE BLOOD AND THEIR CLINICAL SIGNIFICANCE

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In the fifteen years which have elapsed since the first review under the above title was written, an enormous number of publications have appeared which fall under the scope of this title. The space allotted to bring this review (Myers, 1924) up to date permits only a brief summary of a few of the most outstanding contributions since 1924 which have found practical application in clinical work. In general the plan of the first review will be followed, comment being made on the basis of new observations or a new point of view.

Whereas the larger number of blood analyses were originally carried out on whole blood, partly because it was more economical from the standpoint of amount of blood employed and partly because of simplicity, and also historical precedent, there has been a growing realization that the analysis of plasma or serum furnished more reliable information. It soon became quite obvious that the determination of such mineral constituents as calcium, chlorides and inorganic phosphorus required the use of serum (or plasma), while that of pH and CO_2 content required serum or plasma under oil, but this was not so evident in the case of some of the organic constituents which are fairly evenly distributed between cells and plasma. In the case of the very diffusible urea it would not seem to make much difference whether whole blood, serum or plasma was used, but with such organic constituents as sugar, creatinine and uric acid, difficulties in the use of whole blood have gradually come to be appreciated. In the case of sugar, the non-sugar reducing substances are present chiefly in the cells. Hunter and Campbell (1917) early recognized the interference of something present in the cells in the estimation of creatinine, while quite recently Jacobson (1938) has pointed to definite advantages in the use of plasma or serum for uric acid estimations. This indicates that whereas whole

blood may still be used for certain of these determinations, the limitations in its use should be appreciated. No doubt Folin (1930) had some of these facts in mind in introducing his method of so-called unlaked blood analysis, but this method of separating the cells has apparently fallen far short of what Folin had in mind.

In his earliest work on chemical blood analysis Folin introduced the method of recording results in milligrams per 100 cc. of blood, which was soon adopted for most of the determinations. This percentage relationship is probably adequate for most of the organic constituents, but falls short of supplying the most useful information for the inorganic constituents, where equilibrium relationships with each other and other body fluids need to be studied. For this purpose recording values in terms of milliequivalents is particularly helpful, and furthermore variations from normal may be just as readily appreciated as when given in milligrams per 100 cc. (Cf. Peters, 1934; Talbott, 1939).

Blood volume. The dye method of measuring blood volume has recently been reinvestigated with the aid of the colloidal blue dye, T-1824 (Gregersen, Gibson and Stead, 1935), originally described by Dawson, Evans and Whipple (1920). Clinical studies have been made in congestive heart failure by Gibson and Evans (1937), who observed that the change from the compensated to the decompensated state was accompanied by a progressive increase in the volume of plasma and red cells. This increase is shared to a slightly less extent by the plasma than by the corpuscles, resulting in a slight concentration of the blood. In artificial fever Gibson and Kopp (1938) noted a considerable loss of plasma volume in cases where fluid was not given and state that for each individual there is a definite limit beyond which further loss of fluid from the blood stream cannot be tolerated. In 25 cases of hyperthyroidism Gibson and Harris (1939) found the total blood volume increased above normal on an average of 5.45 per cent, while in 7 cases of myxedema it was 15.5 per cent below normal. According to Gibson (1939) pernicious anemia is characterized by a hydremic hypovolemia in the severe stages. In a group of 10 cases the average plasma volume was 30 per cent above, circulating red blood cell volume 68 per cent below, total blood volume 14 per cent below, and total hemoglobin 70 per cent below normal, at a red blood cell level of 1.5 million.

Blood proteins. *Hemoglobin.* The introduction by Van Slyke of an accurate gasometric method of determining the oxygen capacity of blood has made available not only an accurate direct method of es-

timating the hemoglobin, but has also provided a reasonably simple method of checking the standards employed in colorimetric methods. This has resulted in the accumulation of a large amount of data on the hemoglobin concentration of human blood in all parts of the world. The literature has been reviewed by Myers and Eddy (1939) with the presentation of some of their own data on normal subjects. It would appear that normal adults in good nutrition, not residing at excessive altitudes above sea level, show essentially the same concentrations of hemoglobin in the blood, without difference due to race or geographic location. The only difference is that due to sex which has long been recognized. The mean of the hemoglobin values reported for strictly normal individuals is 15.8 grams for the male, 13.8 grams for the female and 12.0 grams for children, per 100 cc. of blood.

Serum proteins. At the time the previous review was written the larger number of serum protein analyses in the literature had been carried out with the aid of the refractometer, and the discussion was based largely on these analyses. Rowe (1917) in particular had extensively employed the refractometric method of Robertson. Further study would indicate that the results obtained with this method were too high, particularly in the albumin fraction, thus leading to relatively high albumin-globulin ratios and somewhat different interpretation of findings. At the present time the method of Howe (1921) is widely used for the determination of the serum proteins and when certain precautions are exercised (Robinson, Price and Hogden, 1937) the values are highly reproducible. The total serum protein of normal individuals generally falls in the range of 6.0 to 8.0 grams per 100 cc. (Fahr and Swanson, 1926; Linder, Lundsgaard, and Van Slyke, 1924; Salvesen, 1926; Wiener and Wiener, 1930; Bruckman, D'Esopo and Peters, 1930; Moore and Van Slyke, 1930; Muntwyler, Way, Binns, and Myers, 1933), with occasional values as low as 5.6 and as high as 8.4. The range of albumin appears to be from 3.6 to 5.4 grams per 100 cc.; however, values as low as 3.4 and as high as 5.6 may be encountered. The range for globulin is 1.5 to 3.4 grams per 100 cc.; however, occasionally the extreme values of 1.35 and 3.55 may be obtained. From the values reported in the literature the range for the albumin-globulin ratio may be given as 1.2 to 2.6. It appears (Bruckman, D'Esopo, and Peters, 1930) that in females the total protein is somewhat higher than in males, due probably to a higher globulin content.

In recent years considerable attention and significance has been given to the serum colloid osmotic pressure (and serum protein concentra-

tion) in studies dealing with the fluid exchange across the capillary membrane and the pathogenesis of various types of edema. The importance of the osmotic pressure of the plasma colloids for fluid exchange in the organism was pointed out by Starling over 40 years ago (see review by Landis, 1934). Epstein (1917) noted that the edema of cases which he originally termed "chronic parenchymatous nephritis," was associated with a deficit of plasma protein. He was the first to apply Starling's theory clinically and suggested that the lowered protein concentration reduces the colloid osmotic pressure of the blood which in turn favors a retention of fluid by the tissues. The results of numerous experimental and clinical studies have amply supported this view and it is now generally accepted that when the serum protein falls below a certain level the tendency to clinical edema increases. Aside from occasional exceptions there is a fairly direct relation between the level of the serum colloid osmotic pressure and the total serum protein concentration. This follows from the fact that serum protein deficits are chiefly deficits of albumin and, further, the albumin exerts considerably more osmotic pressure than the globulin (Govaerts, 1925; Von Farkas, 1926). The following empirical relations between the serum protein and serum colloid osmotic pressure have been suggested:

$$P = C(21.4 + 5.9A) \text{ (Wells, Youmans and Miller, 1933)}$$

where P is the colloid osmotic pressure in millimeters of water, C is the total protein concentration and A is the albumin concentration in grams per 100 cc. and,

$$P = 60.9A + 22.9G - 50 \text{ (Wies and Peters, 1937)}$$

where P also expresses the colloid osmotic pressure in millimeters of water, while A and G represent albumin and globulin in terms of gram per 100 grams serum water.

The relationship between the level of the serum protein and the presence or absence of renal edema was considered by Moore and Van Slyke (1930). In the 75 patients they examined including hemorrhagic, degenerative and arteriosclerotic types of renal disease (cases without cardiac involvement) it was found that when the total protein fell below the lower limit of the range 5.5 ± 0.3 per cent, or the albumin below 2.5 ± 0.2 per cent, edema was usually present. Similar observations have subsequently been made by others and whereas the "critical" protein levels differ slightly those given above are now generally accepted. Of course it must be appreciated that such a relationship cannot be taken as an absolute rule. It is recognized that altered

capillary permeability to protein, sodium chloride administration, increased capillary pressure and other factors may complicate the simple relations.

Serum protein deficits are encountered clinically more often than hyperproteinemia. The estimation of serum proteins in the various forms of renal disease is of considerable value. In the non-hemorrhagic degenerative Bright's disease or nephrosis the serum protein is decreased markedly and this is due almost entirely to a deficit of albumin (Fahr and Swanson, 1926; Linder, Lundsgaard and Van Slyke, 1924; Epstein, 1917; Peters, Bruckman, Eisenman, Hald and Wakeman, 1931; Van Slyke et al., 1930; Salvesen and Linder, 1923). The tendency toward edema formation in nephrosis is closely related to the serum protein (albumin) concentration and hence its determination is very important clinically. In acute glomerular nephritis the plasma protein concentration may remain within normal limits throughout the illness if it runs a mild course (Van Slyke et al., 1930; Peters, Bruckman, Eisenman, Hald and Wakeman, 1932a). Such cases may be accompanied by a rather marked edema despite the fact that the serum protein is within the normal range. This type of case is an outstanding example of the lack of relationship between the serum protein level and the tendency toward edema and one must assume that additional factors participitate in the acute stage (Wiener and Wiener, 1930; Moore and Van Slyke, 1930; Muntwyler, Way, Binns and Myers, 1933; Peters, Bruckman, Eisenman, Hald and Wakeman, 1932a). In the more severe cases and as the condition is prolonged considerable protein deficits may be encountered (Fahr and Swanson, 1926; Van Slyke et al., 1930; Peters, Bruckman, Eisenman, Hald and Wakeman, 1932a). In the terminal glomerular nephritis the proteins are usually at a lower normal value; however, not infrequently the values are somewhat below normal (Van Slyke et al., 1930; Peters, Bruckman, Eisenman, Hald and Wakeman, 1932b). In the arteriosclerotic Bright's disease the serum proteins are generally within normal limits. Lowering of the serum protein which is chiefly at the expense of the albumin fraction is found in patients showing evidence of malnutrition (Bruckman, D'Esopo and Peters, 1930; Bruckman and Peters, 1930; Weech and Ling, 1931) in heart failure (Payne and Peters, 1932) and in cirrhosis of the liver (Myers and Keefer, 1935; Butt, Snell and Keys, 1939). In the latter disease the globulin fraction tends to be increased so that the total protein level may not be disturbed; however, the albumin-globulin ratio is markedly decreased. In hepatic disease it has been reported (Butt,

Snell and Keys, 1939) that there is no constant relationship between the serum protein or serum colloid osmotic pressure and the presence or absence of edema.

Recent literature indicates that hyperproteinemia may not be such a rare occurrence as was once believed. In Kala-azar Wu (1922) noted that the total serum proteins may be increased to 9 or 10 per cent as a result of a rise in the globulin content, the albumin on the other hand being definitely reduced. A similar disturbance of the serum protein and the albumin and globulin fractions appears to be encountered in multiple myeloma (Foord, 1934–35; Feller and Fowler, 1938; Gutman and Gutman, 1937) and in lymphogranuloma inguinale (Gutman and Gutman, 1937). Severe dehydration is accompanied by an elevated serum protein concentration.

Non-protein nitrogenous constituents. *Non-protein and urea nitrogen.* Although the non-protein or urea nitrogen are proably still regarded as furnishing more information of diagnostic or prognostic import so far as the kidneys are concerned than any other single determination, it is recognized that elevated values may be attributed to other causes. That is, non-protein nitrogen retention is experienced not only from impairment in renal function or urinary obstruction but is encountered, for example, in instances of excessive tissue protein catabolism and dehydration. Such disturbances must be borne in mind in evaluating the renal significance of elevated non-protein nitrogen values.

Since urea is the main end product of nitrogenous metabolism, and its excretion one of the kidneys' chief functions, it seems most logical that the kidneys' ability to remove it from the blood should constitute a reliable index of renal function. Twenty-five years ago Ambard tried to demonstrate that more information regarding renal function should be obtainable from data on the urea concentration of the blood, and the amount of urea excreted in the urine, than from either of these factors alone. Although an enormous amount of work was done by Ambard, McLean, Addis and their co-workers, it remained for Van Slyke and his associates to work out an applicable mathematical formula, accurately covering the physiological factors involved, viz., the blood urea concentration and urea excretion in the urine. From their studies Möller, McIntosh and Van Slyke (1928) concluded that the simplest and most satisfactory way to express the relationship between these two factors was by means of the "blood urea clearance" by which term they indicated the *cubic centimeters of blood per minute cleared of urea by renal excretion.* Two formulae were employed, the "standard

blood urea clearance," and the "maximum blood urea clearance," the latter being used only when the volume of urine excreted exceeded 2 cc. per minute. In comparing the blood urea clearance test, with the blood urea and blood creatinine content alone, and the phenosulfonephthalein test, Van Slyke, McIntosh, Möller, Hannon and Johnston (1930) found that the blood urea clearance usually fell below 50 per cent of its normal value before any of the other 3 values showed any abnormality. It was only after the blood urea clearance indicated less than 20 per cent of normal renal function that all values for blood urea and creatinine content, and for phenolsulfonephthalein excretion, were found outside the limits of normal variation. From an extensive study of clinical cases Van Slyke et al. (1930a) concluded that in hemorrhagic and degenerative nephritis one may interpret the blood urea clearance as a measure of the proportion of glomerular tissue still functioning while in arteriosclerotic nephritis the fall in blood urea clearance is proportional probably to the decrease in renal blood flow rather than to the glomerular destruction. The standing which the Van Slyke blood urea clearance test has achieved as a test of renal function is indicated by the fact that it is now quite generally employed as the test of reference.

In 1917 Mosenthal and Hiller suggested the ratio of the urea to the non-protein nitrogen of the blood as an index of the amount of effectively functioning renal tissue, irrespective of the blood urea. Although Killian (1921) effectively employed this ratio to differentiate between the nephritic and non-nephritic toxemias of pregnancy, the test was apparently forgotten until quite recently. Mosenthal and Bruger (1935) have run a large series of determinations on normal and nephritic patients and compared their findings with the Van Slyke blood urea clearance test. They have found that in general the urea ratio rises as the urea clearance values fall. Although the results of the two tests were usually found to be in agreement, they did not entirely parallel each other. However, the discrepancies between the two tests were so slight as to make it certain that if one test is of value the other must be satisfactory. Probably the greatest advantage of the test is that it can be carried out on one specimen of blood, and does not require prolonged observation of the patient or collection of urine.

Uric acid. The uric acid of the blood has continued to receive attention particularly in connection with gout, a rather unexpected finding being the relatively higher and more uniform values for uric acid noted by Talbott, Coombs and their co-workers in serum than in whole blood.

In the most serious modern attempt, up to the time, to elucidate the

pathology of uric acid in gout, Folin, Berglund and Derick (1924) found the uric acid content of whole blood to vary in 9 cases from 5.6 to 10.7 mgm. per 100 cc. They concluded that the unique and characteristic high levels of uric acid in normal human blood are due to a lack of responsiveness on the part of the human kidney, and that this is exaggerated in gout and is the main or only reason why the gouty carry abnormally high levels of circulating uric acid. They observed, further, that the uric acid-destroying process in the gouty is intrinsically about the same as in normal persons, but is subject to wider variations and that the high levels of circulating uric acid in the gouty automatically result in more extensive destruction and, therefore, in diminished excretion (unless the power of destruction is very small). In an effort to obtain a basis for the early differential diagnosis of gout, Hench, van Zant and Nomland (1928) made a clinical comparison of 100 cases each of gout, rheumatic fever and infectious arthritis. The criteria employed for an indisputable diagnosis of gout were, a history of joint disease together with a blood uric acid of 5 mgm. or more for each 100 cc., or tophi, or both. They point out that the three signs which are considered the principal diagnostic points, viz., chronic hyperuricemia, tophi, and punched out areas, are indefinite as to time of appearance but usually appear late. In other words, there need not be a very definite elevation of the blood uric acid in the early stages of the disease. They further observed that renal lesions are highly predominant in association with the arthritis of gout and conclude that the chronic arthritis associated with nephritis is the arthritis of gout until proved otherwise.

The recent investigations of Talbott, Jacobson and their co-workers have given us a new point of view. Their studies indicate that more reliable information regarding blood uric acid may be obtained from serum separated under oil, and that the disturbed metabolism is probably not confined to uric acid alone. In a detailed study of two patients during several attacks of acute gout, Talbott, Jacobson and Oberg (1935) observed the following changes in water and salt metabolism: 1, a diuresis before any clinical or subjective evidence of gout was manifest, and 2, a negative sodium and chloride balance accompanying this diuresis, followed by an increased excretion of potassium, calcium, ammonia, titrable acid, phosphate and urate.

Employing serum separated under oil Jacobson (1938) found the fasting uric acid in 100 non-gouty individuals on a mixed diet to range from 1.9 to 6.7 mgm. per 100 cc., with a mean of 4.2 mgm. In 97 in-

dividuals the serum uric acid was less than 6.0 mgm. In 177 analyses on 21 cases of gout the serum uric acid ranged from 5.2 to 14.8 mgm., 98 per cent of the values exceeding 6.0 mgm. and 94 per cent 7.0. Some evidence of correlation between the onset of an attack, the severity of the disease and the level of serum uric acid was presented. Talbott and Coombs (1938) made a functional study of 24 cases of gout. The serum uric acid values ranged from 5.7 to 14.2 mgm. per 100 cc., the average minimum values being well over 7.0 mgm. They interpreted the increased concentration of uric acid as due to increased formation rather than diminished excretion or destruction. In another study Talbott and Coombs (1938b) examined the serum of 68 non-affected members of families of 16 gouty patients and found that 14 (slightly over 20 per cent) showed concentrations of uric acid greater than 6.0 mgm. They are of the opinion that these findings supported the hypothesis that gout is a familial disease and that one manifestation of it, i.e., an elevation of the serum uric acid, may be subject to hereditary transmission.

In some cases of scarlet fever Voigt and Schülke (1934) have observed blood uric acid figures as high as 20 to 25 mgm. per 100 cc. during the 12 to 24th day of the disease. Voigt (1933) has likewise noted very high figures in nephritis complicated with pneumonia.

Several investigators have objected to the statement originally made by Myers, Fine and Lough (1916) that uric acid was apparently the first of the three waste products, uric acid, urea and creatinine, to become elevated in the blood in renal disease. Johnston (1931) in particular has questioned this conclusion. Bruger and Mosenthal (1932) have carefully reinvestigated this question and state: "In the majority of cases of Bright's disease the uric acid is the first substance in the blood to be augmented with increased impairment of renal function."

Creatinine. Behre and Benedict in 1922 presented evidence to show that creatinine does not exist in normal blood. The facts upon which they made this deduction were: 1, that creatinine can be removed from blood by bone black, but bone black does not affect the chromogenic substance present in blood filtrates, which react with picric acid and sodium hydroxide; 2, that a substance present in picric acid blood filtrates gives a good reaction with sodium carbonate, and this reaction is increased in bloods showing an abnormally high "creatinine" content, whereas pure creatinine in picric acid is only slightly affected by carbonates; 3, that creatinine is readily destroyed by heating in alkaline solution, but the chromogenic substance present in blood is not ap-

preciably affected; 4, that kaolin completely removes small amounts of creatinine from blood, but does not affect the picric acid-alkali reacting substance. Their observations were confirmed, and two additional observations support this view: 1, Benedict and Behre (1936) found that the color obtained on blood filtrates and diluted serum ultrafiltrates with 3,5-dinitrobenzoate differs from that of creatinine, and 2, the apparent creatinine of normal dog serum ultrafiltrates (Gaebler, 1937) and plasma filtrates of human and other species (Behre and Benedict, 1937) fail to precipitate under conditions which precipitate added creatinine. Shortly after the appearance of Behre and Benedict's original paper, Folin (1922) stated in these Reviews: "In view of the many still active investigators who in the past have made contributions to the creatine-creatinine problem one can safely predict that the findings and conclusions of Behre and Benedict will not long remain without contradiction or verification."

Quite as Folin prophesied the observations and interpretations of Behre and Benedict have led to a bitter controversy which has not yet ended. There is no question about the presence of creatinine in urine. It would appear therefore that the kidney must 1, either separate and concentrate creatinine present in the blood, or 2, form creatinine from a closely related precursor compound present in the blood and then excrete it. If the observations of Behre and Benedict are correct the latter would serve to explain the discrepancies. The opponents of Behre and Benedict, however, feel that this explanation is unnecessary. Hayman, Johnston and Bender (1935) found that when trichloroacetic acid (rather than picric acid) was employed as the protein precipitant on serum or plasma, the filtrate differed little in its reactions from pure creatinine. Danielson (1936) came to much the same conclusion from a study of plasma ultrafiltrates. With the aid of a specific bacterial enzyme, Miller and Dubos (1937) have approached the problem in a different way and conclude that in normal individuals creatinine constitutes 80 to 100 per cent of the chromogenic material in serum or plasma.

Gaebler in particular has made important contributions to a solution of this problem. With Keltch (1928) and independently (1930) he demonstrated that creatinine could be isolated from normal blood, and in quantity from retention blood. The creatinine thus isolated was definitely identified chemically. Although originally opposing Behre and Benedict he later agreed (1930) that creatinine as such was not present in detectable amounts in normal blood. Quite recently Gaebler

(1937) and Gaebler and Abbott (1938) have shown that most of the apparent creatinine of ultrafiltrates of normal blood can be precipitated with picric acid and rubidium. Goudsmit (1936) has compared the apparent creatinine content of renal venous blood and of arterial blood, and found the former consistently lower, thus definitely indicating that the apparent creatinine of the blood is the precursor substance of urinary creatinine.

It is evident therefore that the apparent creatinine of blood, whether creatinine itself or a precursor, can be converted in the test tube, in considerable part at least, to creatinine and can be so converted by the kidney. Consequently the apparent creatinine of blood, if not creatinine, has the same significance in blood as creatinine.

In connection with the prognostic significance of high blood creatinine it is of interest that Miller and Dubos (1937) have observed that in plasma from uremic patients there may be large amounts of non-creatinine, chromogenic material, which in several patients seemed to parallel the severity of symptoms of the uremic toxemia.

A number of workers have stated or implied that the retention of creatinine went hand in hand with urea, and therefore the prognostic value of an elevated creatinine was no greater than that of urea or non-protein nitrogen. Bruger and Mosenthal (1932) state in this connection: "It has been appreciated for a long time that creatinine rises in the blood only when renal function becomes markedly impaired (Myers and Lough, 1915). Our results show that normal creatinine values may be found in the blood with as much as 85 per cent of the kidney function lost, as measured by the urea clearance test. When the urea clearance has fallen to about 5 per cent of normal and uremia is impending, the creatinine begins to mount in the blood."

Guanidines. The possible clinical importance of guanidines in the blood has attracted renewed interest since the introduction of a colorimetric method for their estimation in the blood by Major and Weber in 1927. For this purpose they greatly improved the color reagent for guanidines described by Marston (1925). Although guanidine compounds have not been isolated from blood, the utilization of this color reaction has given results which are strongly suggestive of the presence of guanidines, probably methyl guanidine.

In their first papers Major and Weber (1927) described an increase in the "guanidine" content of the blood of certain patients with arterial hypertension. Major (1938) has continued this study and states: "It is of some interest to note that the percentage of increased 'guanidine'

values for the hypertensive patients in this series of 800 patients is approximately the same as that obtained a number of years ago for a smaller group." Pfiffner and Myers (1930) found a small increase in guanidine values in a small series of hypertensive cases with slight or no nitrogen retention. In a series of 49 cases with nitrogen retention Andes, Linegar and Myers (1937) found an increase in guanidines up to ten times the normal, but this appeared to follow the degree of azotemia rather than the hypertension. In a series of 11 cases of hypertension without nitrogen retention the same workers found the blood guanidine scarcely above normal limits.

In 1928 Minot and Cutler presented data which showed that the symptoms of chloroform and carbon tetrachloride poisoning closely resemble those of guanidine poisoning. They noted especially a central necrosis of the liver, and an increase in the blood guanidine. Calcium therapy was found to relieve the symptoms of poisoning and to lower the blood guanidine. Later they (1929) noted an increased guanidine in some cases of liver disease and eclampsia. Further evidence in support of these general findings was presented (Cutler, 1931; Minot and Dodd, 1932). Andes, Andes and Myers (1937) noted increased guanidine values in severe toxemias of pregnancy. Furthermore, the hyperguanidinemia was found to continue as long as the toxic condition existed. Patients in eclamptic convulsions always showed markedly increased guanidine values. In this connection it is of interest that Ellis, Neal and Frazer (1931) found high guanidine values in epileptics, the highest values being near the end of the seizures.

Sugar. As far back as the time of Claude Bernard discussion arose as to the reliability of copper reduction in the determination of blood sugar. With the advent of our modern blood sugar methods, e.g., Bang, Lewis-Benedict, Folin-Wu, the question was again raised as to what percentage of the value obtained was glucose. In 1925 Benedict attempted to prepare a reagent which would be more specific for glucose and described a copper reagent which, when applied to the Folin-Wu tungstic acid filtrate, gave results definitely lower than those obtained by the Folin-Wu method. Almost simultaneously with the publication of Benedict's copper method, Hiller, Linder and Van Slyke (1925) made an important contribution by showing that the residue of reducing substances after yeast fermentation amounted to 10 to 30 mgm. per cent in terms of glucose. The use of yeast in the determination of the non-sugar reducing substances in blood has been employed to advantage by Folin and Svedberg, Somogyi, and Benedict. Folin

and Svedberg (1926) found that with the new Folin method the non-fermentable reducing substances amounted to 5 or 6 mgm., whereas with the Folin-Wu method it was larger, about 22 mgm. Somogyi and Kramer (1928) found that the reducing non-sugar, as determined by the Shaffer-Hartmann method, amounted to about 22 mgm., and that the determination of the true sugar by three different methods yielded essentially identical values. Somogyi (1928) further observed that more than 75 per cent of the non-sugar reducing substances was in the cells, the average value on 36 bloods being 8 mgm. for the serum and 40 mgm. for the corpuscles. Apparently feeling that his first copper reagent fell just short of measuring the true glucose content of the blood, Benedict (1928) elaborated another copper reagent which apparently gives the true glucose content of the blood when applied to the tungstic acid or tungstomolybdic acid blood filtrate. As a result of observations made with this new reagent and of fermentation experiments Benedict demonstrated that the Folin-Wu technique gave figures for blood sugar which averaged about 22 mgm. per 100 cc. of blood too high. Recognizing that the Hagedorn-Jensen (1923) method, which employs a zinc protein precipitant, gave somewhat lower results for blood sugar than most of the other methods, Somogyi (1929) developed a zinc precipitant which completely removes the non-sugar reducing substances. Employing this precipitant, Somogyi found that the Shaffer-Hartmann (modified), Folin-Wu, Folin and Benedict's second copper method gave essentially the same results. Although the nature of the non-sugar reducing substances has not been entirely solved, it would appear from the work of Benedict and Newton (1929) that glutathione constitutes much the largest part of the non-sugar reducing substances. They do not believe that the thionine present ordinarily accounts for more than 1 or 2 mgm. of reduction per 100 cc. of blood, although occasionally it might amount to 4 or 5 mgm.

In blood sugar estimations giving normal or elevated values, it probably is not necessary to employ methods yielding true blood sugar values, but with hypoglycemia it is important to know the true glucose content. This is illustrated in the case of hyperinsulinism reported by Murphy, Duslin and Bowman (1939) where fasting blood sugars ranged between 20 and 30 mgm. per 100 cc. with the Benedict (1928) method over a ten day period. Space does not permit discussion of blood sugar variations in disease but it may be pointed out that there is a fuller clinical appreciation of the useful information obtainable with the glucose tolerance test.

Blood lipids. (*Fat, phospholipids, cholesterol.*) Although many new and improved methods for the estimation of the blood lipids (Bloor, 1928, 1929; Man and Gildea, 1932; Man and Peters, 1933; Kirk, Page and Van Slyke, 1934; Schoenheimer and Sperry, 1934; Thannhauser and Setz, 1936) have been introduced during the past fifteen years and an extremely large number of investigations have been carried out, relatively few new facts of direct clinical importance have been uncovered. In general the fundamental facts regarding fat metabolism outlined by Bloor with the aid of his older methods have been substantiated. Since the blood lipids have been the topic of recent reviews (Sinclair, 1937) they will be discussed only briefly here.

The changes which occur in normal pregnancy, diabetes, nephrosis and pernicious anemia are well known and were previously discussed.

Hurxthal (1933a) in particular has shown that the plasma cholesterol may be of considerable clinical value in thyroid disease. Cholesterol was found to be low in toxic thyroid states, the lowest values being found in patients in or near thyroid crises. In this respect it bore a reciprocal relation to the basal metabolism. Hurxthal (1933b) found that the cholesterol could be brought to a normal level partly by preoperative treatment but chiefly by subtotal thyroidectomy. Postoperative myxedema, on the other hand was found to be accompanied by hypercholesterolemia (Hurxthal, 1934). Subtotal thyroidectomy in some cases was followed by hypercholesterolemia without clinical myxedema. This was interpreted as a transient thyroid deficiency. The finding of hypercholesterolemia, in the absence of its few other common causes, is believed to point more specifically to thyroid deficiency than the finding of a low basal metabolism.

E. Z. Epstein (1931, 1932), continuing the studies begun by Rothschild on cholesterol in liver disease, has emphasized in particular the clinical value of cholesterol partition. It should be noted that in 1926 Thannhauser and Schaber first recognized the clinical importance of the disturbed relation of cholesterol and cholesterol esters in liver disease, and attributed the drop in cholesterol esters in hepatic damage to a disturbance in liver synthesis and hydrolysis. Epstein and Greenspan (1936) have observed that in obstructive jaundice hypercholesterolemia is usually encountered, affecting both the free and ester fractions, which parallels the degree of hyperbilirubinemia. In jaundice occurring in acute degeneration of the liver blood cholesterol did not rise with the bilirubin, but usually remained normal or subnormal. The cholesterol ester was usually lowered in acute degeneration of the liver and mirrored the severity of the damage. In rapidly fatal cases the ester

was low or even absent throughout the course of the disease. Repeated cholesterol partitions in liver and biliary tract disease during the course of the illness indicated the trend of the disease. The recent work of Shay and Fieman (1938) confirms the observations of Epstein, as do those of Boyd and Connell (1938), who also give data on the total lipids.

In the active stages of chronic hemorrhagic nephritis, Page, Kirk and Van Slyke (1936) observed a tendency to lipemia, with the plasma lipids near or above the upper limit of normal. As the disease passed into the terminal stages the lipemia was likely to decrease and the plasma lipid to fall below normal before exitus. The individual lipid constituents, free cholesterol, cholesterol esters, phosphatides, and the neutral fat fraction were found to rise and fall together. In eclampsia Boyd (1936) has observed a high phospholipid content with a low cholesterol, and a particularly high phospholipid-cholesterol ester ratio.

Man and Peters (1934) have shown that in diabetic acidosis cholesterol falls below the acidosis level during the period immediately following the disappearance of acidosis and dehydration, and that at this time the cholesterol content of the blood may be even lower than at the end of convalescence.

The blood lipids have been studied by Man and Gildea (1936) in malnutrition, and in most cases were found to be reduced. They suggest that part of the hypoproteinemia and hypolipemia associated with debilitating diseases such as nephritis, tuberculosis and possibly hyperthyroidism, may be attributed to the state of nutrition.

Serum pigments. (*Bilirubin, carotene.*) Although it has long been appreciated that the light golden yellow color of normal blood serum was due chiefly to *bilirubin*, it was only comparatively recently that quantitative methods of estimating the bilirubin or the degree of yellow color have come into common clinical use. As early as 1913 Hymans, van den Berg and Snapper employed the Ehrlich's diazo-reaction for the estimation of bilirubin, while Meulengracht (1920) first compared the blood serum, after suitable dilution with physiological salt solution, with 1:10,000 potassium dichromate, thus obtaining the so-called icterus index. Hoover and Blankenhorn (1916) were the first investigators in this country to utilize the chemical examination of blood plasma in the study of jaundice. Maue (1922) first employed the icterus index test, but Bernheim (1924) deserves the credit of first utilizing the icterus index in an extended clinical study. This test and the van den Bergh reaction, in several modifications, have since found extensive clinical use in this country.

Bilirubin is not the only yellow pigment present in serum, as carotene

may occasionally constitute a considerable proportion of the total color. Hess and Myers (1919) were the first to call attention to the occurrence of carotenemia. More recently Nation and Myers (1934) studied the possible influence of carotene on the icterus index in 161 cases, and concluded that carotenemia does not alter the validity of the icterus index, except in cases of marked carotenemia such as may be found in some diabetic patients, and then only if the icterus index is quite low.

At the present time there are two schools, one preferring to use the simple icterus index test, the other the quantitative (and qualitative) van den Bergh reaction. When only the relative amount of bilirubin is desired the estimation of the icterus index is a perfectly satisfactory clinical procedure. If the van den Bergh reaction or any of its modifications had been proven to furnish a simple and fully accurate quantitative method of determining the milligrams of bilirubin per 100 cc. of blood serum, this would be the method of choice, but as yet such does not appear to be the case. Obviously the icterus index is not an exact procedure owing to the presence of other pigments in the serum, chiefly carotene, but the work of White (1933) has given the test quantitative significance, since he has shown that an icterus index of 6 matches roughly a synthetic jaundice serum containing 1 mgm. of bilirubin per 100 cc. The chief objection to the quantitative van den Bergh reaction centers around the question of the completeness of the extraction of bilirubin from the serum. This was greatly improved by the Thannhauser and Andersen (1921) modification and by that of White (1932), but there is still some question that the extraction is 100 per cent quantitative.

Although authorities differ, the normal serum bilirubin would appear to vary from 0.5 to 1.5 mgm. per 100 cc., but may exceed 40 mgm. in severe forms of jaundice. The normal range of the icterus index is given as 4–6, latent jaundice above 10, while severe jaundice may exceed 225.

Much use has been made of the qualitative van den Bergh test because the direct and indirect reactions were supposed to differentiate between the obstructive and hemolytic type of jaundice. Bilirubin which has passed through the epithelial cells of the liver, but is not excreted due to obstruction of the bile ducts, yields a "direct" reaction when it is regurgitated into the blood stream. In contrast to this, bilirubin which has not yet passed through the liver cells, yields a delayed or "indirect" reaction. Where the reaction is direct, one of

two types of pathological change has occurred. Either there is obstruction of the larger bile channels from within or without, or else extensive diffuse necrosis of the parenchymal cells has taken place. The pathological explanation for "indirect" reacting bilirubin resides in the fact that the polygonal cells are not capable of excreting into the bile channels all of the pigment which is brought to them. The "biphasic" reaction is probably due to the simultaneous presence of bound and free bilirubin in the blood stream. Pathologically it would indicate both some degree of obstruction of the bile canaliculi and some diffuse alteration of the parenchymal cells.

White (1933) has obtained valuable data bearing on the amount of bilirubin bound by the serum protein, by determining the icterus index before and after precipitating the serum proteins with alcohol, the latter being named the residual icterus index. In this way the icterus index can be made to give some of the information furnished by the qualitative van den Bergh reaction.

Chiefly as a result of the remarkable series of studies by Mann and his co-workers (1924) it has been shown that bile pigment formation continues after the removal of the liver, and indeed, as Rich (1925) has shown, after all the abdominal viscera have been removed. The reticulo-endothelial system (especially the bone marrow and spleen) is mainly concerned with this process. Although the Kupfer cells of the liver belong to this system, they are of little or no importance in the production of bile pigment. Apparently the function of the liver in bile pigment metabolism is not to secrete the bilirubin, but to excrete it, and the liver would appear to be the only source of removal of bilirubin from blood to bile. Practically, the excretion of bilirubin by the liver may be compared to the excretion of urea by the kidney, and may therefore furnish valuable information regarding liver function.

In its simplest terms hyperbilirubinemia may be due either to an increased formation of bilirubin (*hemolytic jaundice*) or to a decreased excretion of bilirubin either as a result of obstruction (*obstructive jaundice*) or due to a deficiency in the bilirubin excretory function of the polygonal liver cells (*hepatogenous jaundice*). Conversely hypobilirubinemia is due to a decreased formation of bilirubin, and is quite generally found in the secondary anemias.

Acid-base balance. The cation content of the serum is made up almost entirely of sodium, potassium, calcium and magnesium while the anion content is made up of chloride, bicarbonate, phosphate, protein and a small amount of sulfate and organic acid. Since at the

normal blood reaction (pH 7.4) the cations exist in combination with acids as neutral salts the milliequivalent per liter concentrations can be related as follows:

Total base	= Na	+ K	+ Ca	+ Mg	= Cl	+ HCO_3	+ Pr	+ PO_4	+ R
154	= 142	5	5	2	= 105	25	17	2	5

The concentrations indicated represent the findings of a number of workers (Kramer and Tisdall, 1922; Gamble, Ross and Tisdall, 1923; Peters, Bulger, Eisenman, and Lee, 1926; Oard and Peters, 1929; Keys, 1936) and agree with those obtained in the authors' laboratory. It should be pointed out that the value for R (representing sulfate and organic acid) is obtained by difference and would be expected to be positive. Not infrequently, however, the value has been found to be negative (Atchley and Benedict, 1930–31; Peters, Wakeman, Eisenman and Lee, 1928–29; Hald, 1933). Several workers (Hald, 1933; Sunderman, 1930–31; Hald and Eisenman, 1937) have obtained a lower value for the total base concentration than that given above and the difference appears to be the result of a lower average value for the sodium content. Such data point to an average total base concentration close to 147.0 mEq. and to an average sodium content close to 135.0 mEq. By accepting these values as correct it is apparent that the value for R would be negative; however, it is highly unlikely that this should be the case. In attempting to find an explanation for this discrepancy one immediately suspects that the calculation of the base equivalent of the serum proteins gives values which are too high. Such an error undoubtedly occurs in certain sera with marked divergence of the normal protein content (Gutman, Gutman, Jilson and Williams, 1936); however, for normal sera this is probably not the case. The suggestion has been made (Peters, 1935) that possibly a fraction of the anions is bound to protein. Whereas the problem cannot be answered at the present time, it should be pointed out that Peters and Man (1934) have presented evidence for the existence of lipid-chlorine in serum and were able to explain in part the apparent discrepancy.

For an accurate evaluation of the disturbances of the acid-base balance encountered in various diseased conditions, and as an aid in therapy, it is desirable to include determinations of the serum pH, the total base concentration and the concentration of the various anions (especially chloride and bicarbonate). However, as will become apparent below, very helpful information can be obtained from estimations

of only the serum pH and bicarbonate content. Numerous publications might be cited to establish the normal ranges of the serum pH, CO_2 content and CO_2 tension. The normal values for venous plasma (or serum) as obtained by a number of workers prior to 1926 have been summarized by Austin and Cullen (1926) and the limits observed were reconsidered by Earle and Cullen (1929). These results indicate that the normal range is for pH, 7.30 to 7.52, for CO_2 content, 54 to 74 volumes per cent, and for CO_2 tension, 34 to 62 mm. of mercury. The upper range of pH as given by Earle and Cullen (1929) is undoubtedly too high. In establishing this value the colorimetric method was used upon serum, employing a colorimetric correction of 0.23 pH to correct the values obtained at 20°C. to body temperature. It was not appreciated until later (Robinson, Price and Cullen, 1933; Myers, Muntwyler, Binns and Danielson, 1933) that the colorimetric correction for serum is 0.30 instead of 0.23 pH which is the correction for plasma. Hence it may be assumed that the pH values reported by Earle and Cullen are on the average 0.07 pH too high. Shock and Hastings (1934) have reinvestigated the variation of the acid-base balance in normal individuals and have obtained evidence which indicates that a slight difference exists between the sexes in the average levels of pH, bicarbonate content and carbon dioxide tension. These authors concluded that in males the variation may be from pH 7.35 to 7.45; $(BHCO_3)_s$ from 23 to 30.0 mEq. per liter and pCO_2 from 40 to 50 mm. of mercury, while the variation for females may be from pH 7.37 to 7.47; $(BHCO_3)_s$ from 22 to 28 mEq. per liter; and pCO_2 from 36.5 to 46.0 mm. of mercury. Studies of the variations of individuals from day to day showed that in some persons the fluctuations were as great as the variation between individuals, while in others they were comparatively slight. No characteristic diurnal variations were noted. Attention should be called to the fact that the pH values as ordinarily determined are probably on the average 0.03 to 0.05 pH lower than that existing in the circulating blood. Havard and Kerridge (1929) while measuring the pH of whole blood at 38°C. by means of the glass electrode observed a decrease of pH averaging about 0.05 pH which occurred very shortly after the blood had been drawn. That a fall of pH of approximately this magnitude takes place over a period of about one-half hour after the blood has been withdrawn has been confirmed by a number of workers (Laug, 1930, 1934; Platt, 1936–37). However, the change does not occur abruptly as was first described, this observation being due probably to a "temperature artefact" (Platt, 1936–37), but occurs

gradually as a part of a continuous glycolytic process (Laug, 1934; Platt, 1936–37).

The emphasis of pathways of displacement of the acid-base balance rather than conditions is of distinct advantage. It is now well recognized that there are four major pathways of disturbance of the acid-base balance which are the result of either a respiratory or a metabolic process (Peters and Van Slyke, 1931; Shock and Hastings, 1935). Thus the metabolic type includes both primary alkali excess and primary alkali deficit while the respiratory type includes both primary carbon dioxide (H_2CO_3) excess or primary carbon dioxide deficit. In order to establish a given pathway one must consider the magnitude of the carbon dioxide tension in addition to pH and bicarbonate content. Graphical representation of these three variables can be made by a logarithmic plot (Peters and Van Slyke, 1931), or by employing triaxial coördinate paper as suggested by Hastings and his co-workers (Shock and Hastings, 1934; Shock and Hastings, 1935; Hastings and Steinhaus, 1931). These authors called attention to the fact that triaxial cross section paper is usually ruled with equal spacings between the lines and at a 60′ angle. It is thus adapted to plotting data related by the equation, $ax - by + cz = k$. Since the Henderson-Hasselbalch equation by rearranging takes the form, pH $-$ log $BHCO_3$ + log $H_2CO_3 = pk'$; it is evident that the pH, log $BHCO_3$ and log H_2CO_3 can be plotted arithmetically on the three axes of such paper. These authors point out that the advantage of this method of plotting the data is that constant H_2CO_3 (or CO_2 tension) constant pH, and constant $BHCO_3$ lines are rectilinear and are inclined at equal angles to each other. It is readily possible, therefore, to determine the extent of participation of either the respiratory or the metabolic factors in causing a given path of displacement.

Space does not permit a discussion of the disturbances of the acid-base balance which are encountered in various diseased conditions, consequently the reader is referred elsewhere (Peters and Van Slyke, 1931; Van Slyke, 1934). It is only possible to indicate briefly the pathways of disturbance and to list some of the clinical conditions in which the disturbances are encountered. *a.* Primary alkali deficit (metabolic acidosis) is encountered frequently in pathological conditions. The disturbance may be the result of an excess of fixed acid arising from overproduction or faulty elimination, or the result of an excessive loss of fixed base from the blood. It is encountered, for example, in diabetes, renal disease with nitrogen retention, diarrhea of

infancy and bichloride of mercury poisoning. *b.* Primary alkali excess (metabolic alkalosis) may occur as a result of an excessive increase of the base concentration in the blood unaccompanied by an equivalent increase of acid ions or may occur as the result of an excessive loss of acid ions from the body unaccompanied by an equivalent loss of base. Such a disturbance is encountered following the excessive administration of alkali or as a result of profuse vomiting. c. Primary carbon dioxide deficit (respiratory alkalosis) occurs when the volume of air respired is in excess of that required to maintain the normal ratio of bicarbonate and carbonic acid in the blood. Respiratory alkalosis is encountered during acute clinical fevers, encephalitis, application of hot baths, or on exposure to dry hot air. *d.* Primary carbon dioxide excess (respiratory acidosis) occurs in conditions wherein the blood cannot be freed of carbon dioxide properly resulting in a disturbance of the bicarbonate carbonic acid ratio. It is encountered in emphysema, morphine narcosis and tracheal obstruction.

Mineral constituents. The mineral constituents of the blood serum have been intensively studied in recent years and it has been shown that in normal individuals their concentration is maintained within comparatively narrow limits. It has been recognized for many years that sodium is found chiefly in the body fluids, while potassium is a constituent principally of the cellular tissue. Recent work has firmly established this fact and since they represent the principal cations of these phases their importance in controlling osmotic pressure levels is clearly recognized. Such interesting subjects as the electrolyte distribution in body fluids and cells, and transfers of water and solutes in the body, are outside the scope of the review and the reader is referred elsewhere (Peters, 1935; Fenn, 1936; Darrow and Yannet, 1935; Harrison, Darrow and Yannet, 1936; Hastings and Eichelberger, 1937).

Sodium. Sodium represents approximately 92 per cent of the total base content of serum. In the past the usually accepted normal range for serum sodium was taken to be 138 to 148 mEq. per liter, the values being based largely upon the observations of Kramer and Tisdall (1922). More recent determinations (Dill, Talbott, and Edwards, 1930; Hald and Eisenman, 1937; Keys, 1936; Sunderman, 1930–31) indicate that these values are somewhat high. For example, Hald and Eisenman (1937) from their series report the following range, 129.1 to 139.2 mEq. with an average of 135.1 mEq. per liter. The human erythrocyte contains considerably less sodium (Dill, Talbott and Edwards, 1930; Hald and Eisenman, 1937; Keys, 1936; Oberst, 1935; Crabtree, and

Maizels, 1937), there being on the average approximately 16.5 mEq. per liter of cells.

Serum sodium deficits are frequently encountered in diseased conditions and dehydration is an accompanying symptom. In 1932 Loeb called attention to the fact that a marked decrease of the serum sodium occurs in the crises of Addison's disease and shortly later he and his collaborators (Loeb, Atchley, Benedict and Leland, 1933) clearly demonstrated that a similar change takes place following double adrenalectomy in dogs. Their experiments indicated that a marked urinary loss of sodium occurs, the urine sodium being increased in amount and concentration. Other electrolyte disturbances noted were decreases of the chloride and bicarbonate content, which together approximately equaled the decrease of sodium, and an elevation of the potassium value. These demonstrated losses of sodium and chloride from the blood offered an explanation for the early recognized benefit of injecting sodium chloride in the treatment of the characteristic crises of Addison's disease. Since sodium deficits are greater than those of chloride it has been suggested (Wilder, Kendall, Snell, Kepler, Rynearson and Adams, 1937; Harrop, Soffer, Nicholson and Straus, 1935) that a mixture of sodium chloride and sodium bicarbonate or sodium citrate (to furnish extra sodium ion) would be more effective in maintaining a normal blood electrolyte concentration. That animals following double adrenalectomy and patients with Addison's disease are sensitive to the potassium intake is generally recognized and the importance of controlling the mineral composition of the diet in this disorder was thoroughly emphasized by Wilder, Kendall et al., (1937). Many publications have appeared in recent years dealing with the electrolyte and water and other changes which occur following adrenalectomy in various animals and in Addison's disease. A number of hypotheses have been presented to explain the symptoms of insufficiency and at the present time there is no unanimity of opinion. Various phases of the subject have recently been discussed by a series of authors in the Cold Spring Harbor Symposia on Quantitative Biology, volume 5, 1937.

In addition to Addison's disease the sodium is found lowered in other clinical conditions accompanied by dehydration. Thus a lowered serum total base (sodium) content is associated with the dehydration encountered in upper intestinal tract obstruction with profuse vomiting (Gamble and Ross, 1925; McIver and Gamble, 1928), pancreatic fistula (McIver and Gamble, 1928), terminal nephritis (Peters, 1932), diabetes (Peters, Kydd, Eisenman and Hald, 1933), bichloride of mercury poison-

ing (Talbott, Coombs and Consolagio, 1937), and diarrhea (Marriott and Hartmann, 1928).

Potassium. The serum potassium of normal individuals appears to vary between 3.0 and 7.0 mEq. per liter (Dill, Talbott, and Edwards, 1930; Kramer and Tisdall, 1921, 1922; Hald and Eisenman, 1937; Keys, 1936; Sunderman, 1930–31), with an average value close to 4.5 mEq. Potassium is the principal cation of the human erythrocyte and Hald and Eisenman (1937), who obtained values somewhat lower than other workers (Dill, Talbott and Edwards, 1930; Keys, 1936; Kramer and Tisdall, 1921, 1922) give as an average value 82.5 mEq. per liter with variations from 71.8 to 101.7 mEq. per liter.

The observation that the serum potassium is elevated in animals following double adrenalectomy (Hastings and Compere, 1931), and in patients with Addison's disease (Loeb, 1932, 1933) has renewed interest in the serum potassium changes in other clinical conditions. On the whole, the serum potassium changes which have been noted in other clinical disorders are not very great and this emphasizes the necessity of carefully controlled observations. Rabinowitch (1924–25) noted in several of his cases of advanced renal disease that the serum potassium was elevated. Hoffman and Jacobs (1933–34) studied the serum potassium in a large series of individuals including a variety of diseases and the value was found to be altered appreciably only in renal disease and asthma. In renal disease the serum potassium was usually elevated (maximum elevation to 9.8 mEq. per liter) whenever there was marked nitrogen retention. These findings are complicated, however, since certain cases had been given potassium salts. The latter authors observed seven cases of allergic bronchial asthma before and after epinephrine injection. In every case the serum potassium was slightly above what was regarded as normal and became lowered following epinephrine injections (the lowest value was 3.8 mEq. per liter). Recently Rusk, Weichselbaum and Somogyi (1939) also reported evidence for an elevation of the serum potassium above normal during asymptomatic periods and in acute attacks of bronchial asthma. These same authors, who called attention to their earlier findings of definite improvement in six cases of chronic urticaria when receiving low sodium, high potassium, acid ash diets, found evidence for slightly elevated serum potassium values in a group of twenty patients with acute and chronic urticaria. With subsidence of the clinical symptoms the value was found to become decreased. The observation of Briggs, Koechig, Doisy and Weber (1924) that when insulin is administered to normal

animals (dogs) there is a simultaneous decrease in the concentration of glucose, inorganic phosphate and potassium has been confirmed (Kerr, 1928), and the same response occurs following insulin administration in diabetics (Harrop and Benedict, 1924) and normal humans (Rusk, Weichselbaum and Somogyi, 1939; Harrop and Benedict, 1924). The administration of glucose and levulose to dogs (Flock, Bollman, Mann and Kendall, 1938) and glucose to normal individuals (Rusk, Weichselbaum and Somogyi, 1939) is accompanied by a fall of serum potassium. The decrease of serum potassium in dogs following insulin administration could not be accounted for by a gain of erythrocyte potassium (Kerr, 1928). A high serum potassium has recently been demonstrated in experimentally induced intestinal obstruction (Cutler and Pijoan, 1937; Scudder, Zwemer, and Truszkowski, 1937) and has been suggested as a toxic factor in this condition.

Calcium. The serum calcium has received consideration in recent issues of these Reviews: The parathyroid glands—D. L. Thomson and J. B. Collip (1932), and Occurrence, transport and regulation of calcium, magnesium and phosphorus in the animal organism, C. L. A. Schmidt and D. M. Greenberg (1935), The blood calcium and the factor in blood coagulation, J. H. Ferguson (1936). A striking feature regarding serum calcium is the constancy of the values obtained in normal individuals (Mull and Bill, 1933; Kirk, Lewis, Jr., and Thompson, 1935) and in most diseased conditions. The normal range of variation can be taken to be 4.5 to 5.7 mEq. per liter, with an average close to 5.2 mEq. As indicated by the observations of Abderhalden which were made about 40 years ago, recent analyses have made it increasingly evident that the calcium of blood is practically all to be found in the plasma, there being little or none in the erythrocytes. For example, Hald and Eisenman (1937) reported the following range of calcium in cells, namely, −0.5 to 1.4 mEq. per liter with an average of 0.2 mEq.

It has been recognized for some time that clinically significant changes of the serum calcium level are encountered in parathyroid disorders, i.e., hyperparathyroidism being accompanied by elevated values while hypoparathyroidism is accompanied by lowered values. Other conditions where elevated values have been encountered are instances of overdosage with viosterol and in certain cases of hyperproteinemia while lowered values have been reported in certain cases of rickets, infantile tetany, osteomalacia, chronic glomerular nephritis (with high blood phosphorus) and nephrosis (with markedly reduced serum protein). The recent work (McLean and Hastings, 1935) which has demonstrated that total serum calcium, calcium ions (Ca^{++}) and total serum proteins

are related in a manner which can be stated in an equilibrium equation and, further, the evidence of a reciprocal relationship between the levels of serum calcium and phosphorus (Peters and Eiserson, 1929) have aided materially in a better evaluation of the serum calcium clinically.

A large amount of literature has accumulated on the subject of the state of calcium in the body fluids, a review of which will be found elsewhere (Thomson and Collip, 1932; Schmidt and Greenberg, 1935; Ferguson, 1936). Suffice it to state that since the work of Rona and Takahashi (1911), who showed by compensation dialysis that a larger part of the calcium in serum is in a diffusible form, whereas the remaining fraction is not diffusible, it has been customary to distinguish between diffusible and non-diffusible calcium of serum. These authors suggested that the non-diffusible fraction is bound to serum protein, a view which has generally been accepted. As a result of certain solubility studies they (Rona and Takahashi, 1913) concluded that the diffusible calcium represents calcium bicarbonate in a metastable supersaturated solution. Subsequent work by a number of investigators has led to the belief that diffusible calcium is in part ionized and in part bound in some unionizable form. The development by McLean and Hastings (1934) of the biological method of directly estimating the calcium ion concentration of solutions has obviously been of great value in studies of this problem. McLean and Hastings (1935) were able to show that calcium proteinate dissociates as a weak electrolyte and that ionized calcium in protein containing fluids (serum) depended upon an equilibrium between protein and calcium, described as a first approximation by the equation,

$$\text{Ca Prot.} \rightleftarrows \text{Ca}^{++} + \text{Prot.}^{-}$$

and by the equilibrium equation,

$$\frac{(\text{Ca}^{++}) \times (\text{Pr}^{-})}{(\text{Ca Prot.})} = K_{\text{Ca Prot.}}$$

The value of $K_{\text{Ca Prot.}}$, in the case of the combined serum proteins (temperature 25°C and pH 7.35) was found to be $10^{-2.22 \pm 0.07}$. Somewhat different values have been ascribed to $K_{\text{Ca Prot.}}$ by other authors. See for example McLean (1938) and Miller (1937).

The experiments of McLean and Hastings (1935) led to the conclusion, therefore, that the calcium of serum is present almost entirely as calcium ions and calcium bound to proteins and, further, ionized

calcium and diffusible calcium are essentially equal. That the fluids of the body contain a small amount of bound but diffusible calcium was recognized, it being estimated to be 0.15 mM. per kilo of water or less.

Since the serum calcium ion concentration is of primary clinical importance, it is of interest to enquire whether in sera from patients with diseases known to have changes in total serum calcium, the calcium ion concentrations calculated with the aid of the above statement are in satisfactory agreement with the values obtained by direct determination or by ultrafiltration methods. Such comparisons have been made (McLean and Hastings, 1935; McLean, Barnes, and Hastings, 1935; Morison, McLean and Jackson, 1938) and on the whole the findings are highly encouraging. It appears probable, therefore, that as an aid in evaluating changes of serum calcium clinically, one can calculate (with a few possible exceptions) the calcium ion concentrations from the total calcium and serum protein values. In the various clinical conditions studied by McLean and Hastings (1935) evidence for elevated serum calcium ion concentration values (1.37 to 2.0 mM. per liter) was found in hyperparathyroidism. Although the condition was not investigated, elevated values were also indicated for instances of overdosage with viosterol. Lowered values (0.37 to 1.0 mM per liter) were encountered in hypoparathyroidism and in hyperphosphotemia of nephritis while normal values (1.06 to 1.31 mM per liter) were observed in cases of rickets, Paget's disease, senile osteoporosis and calcinosis universalis. Cases of hyper- and hypo-proteinemia were not investigated, however, on the basis of reported values for diffusible calcium, the authors anticipated normal levels in these disturbances. These authors emphasize the importance of the parathyroid hormone in maintaining the calcium ion concentration at a normal physiological level and conclude that an increase in the serum calcium ion concentration is presumptive evidence of hyperfunction of the parathyroid glands. Abnormally low calcium ion concentration values presumably point to a hypofunction of the parathyroid glands. A possible exception to the latter is recognized in the so-called low calcium rickets, associated with infantile tetany.

Magnesium. The magnesium content of normal human serum has recently been investigated by a number of workers (Hald and Eisenman, 1937; Watchorn and McCance, 1932; Greenberg, Lucia, Mackey and Tufts, 1933; Walker and Walker, 1936). It appears that the values of Hald and Eisenman (1937) (minimum 1.2, maximum 2.2 and average 1.6 mEq. per liter) represent the lowest while those of Greenberg et al.

(1933), (minimum 1.6, maximum 2.9 and average 2.3 mEq. per liter) represent the highest values reported. The normal range as reported by the other workers appears to fall in between these values. The red blood corpuscles contain appreciably more magnesium than is found in serum. Hald and Eisenman (1937) reported the following values, namely, minimum 3.5, maximum 6.2 and average 4.6 mEq. per liter, while Greenberg et al. (1933) obtained the somewhat higher average value of 5.4 mEq. per liter.

There appears to be some confusion in the literature regarding serum magnesium changes in various diseased conditions. Apparently the serum magnesium may vary greatly under clinical conditions. Elevated and lowered values have been reported. However, such changes are not uniformly encountered and sometimes in a given diseased condition both high and low values have been experienced. Walker and Walker (1936) studied a large group of hospital cases and found evidence of slightly increased values in cases of hypertension without signs of kidney damage. In five cases associated with greater or lesser degrees of renal damage high serum magnesium values were encountered (the highest value of 3.5 mEq. being observed in a case of terminal nephritis). These authors concluded that the serum magnesium may be elevated in moderate or severe renal insufficiency, especially if associated with hypertension. Hirschfelder (1934) also noted elevated values in some cases of renal disease while in others lowered values were observed. The latter author found that in renal disease, apparently because of decreased excretion, an ordinary purgative dose of magnesium sulfate causes a marked elevation of serum magnesium. Such elevations (to approximately 8.0 mEq. per liter) were accompanied in a number of patients by drowsiness or even light coma. Hirschfelder (1934) observed, in addition to four cases of glomerular nephritis, lowered serum magnesium values in two cases of parathyroid tetany and in three patients suffering from idiopathic epilepsy. It is stated that the low serum magnesium encountered clinically was accompanied by muscular twitchings or by convulsions and when it occurs in renal insufficiency the symptoms may be relieved by the oral administration of epsom salt. Greenberg and Aird (1938) were unable to detect any change of the serum magnesium from normal in epilepsy.

Chlorides. Chloride represents the principal anion of serum and may be taken to vary normally between 100 and 110 mEq. per liter, an average value being close to 105 mEq. Since the red blood corpuscle membrane is permeable to the chloride ion, the chloride content of the

cells is related to that of the serum in accordance with the Gibbs-Donnan law (Van Slyke, Wu and McLean, 1923). At a normal blood reaction (pH 7.4) and complete oxygenation of hemoglobin the molal ratio $(Cl)_c/(Cl)_s$ is approximately 0.7, an average cell chloride concentration being in a round number 50.0 mEq. per liter.

In considering serum chloride changes in diseased conditions it should be appreciated that there is a marked tendency for reciprocal alterations of the bicarbonate and chloride concentrations. Apparently this and other related changes of the serum chloride and bicarbonate take place in an attempt to maintain a normal total electrolyte content. Hypochloremia is encountered much more frequently than is hyperchloremia. It is now generally recognized that profuse vomiting from upper intestinal tract obstruction (McVicar, 1925) or from other causes (Haden and Guffey, 1924) may be accompanied by marked serum chloride deficits. The serum bicarbonate becomes elevated to a varying degree depending upon the extent of total base loss from the body. In renal disease the serum chloride values are somewhat variable. In terminal nephritis lowered values are encountered frequently, sometimes to a rather marked extent, and this alteration appears closely associated with nausea, vomiting and anorexia (Peters, 1932). Values at the upper range of normal or somewhat above are frequently observed in the acute and nephrotic forms of glomerular nephritis. Serum chloride deficits are quite regularly encountered in diabetic acidosis (Peters, Kydd, Eisenman and Hald, 1933), and unusually low values may be observed in acute mercuric chloride poisoning (Muntwyler, Way, and Pomerene, 1934). In Addison's disease (Loeb, Atchley, Benedict and Leland, 1933), lowered chloride values are associated with serum sodium deficits. In diarrhea of infancy somewhat variable findings for the serum chloride have been reported (Hartmann, 1928; Hamilton, Kajdi and Meaker, 1929); however, most frequently the values appear normal or are slightly elevated. In this condition the loss of base in excess of chloride in the diarrheal stool gives rise to the acidosis and dehydration which is encountered. Among the other clinical conditions wherein lowered serum chloride values are encountered are lobar pneumonia, various conditions involving improper ventilation of the blood, fevers, tuberculosis and pleural effusion.

Phosphorus. This brief discussion is limited to the normal and abnormal variations of the serum inorganic phosphate only and the values are expressed in terms of milligrams per 100 cc. rather than mEq. per liter. It has been customary to regard the normal range of varia-

tion of serum inorganic phosphate to be, in adults, from 3 to 4.5 mgm. per 100 cc. and in children from 4 to 6 mgm. per 100 cc. The recent observations of Stearns and Warweg (1933), who studied the partition of phosphorus in blood and serum in normal individuals from birth to maturity, are of interest in this connection. It was found that the *serum inorganic phosphorus rises steadily after birth until the maximum* value (approximately 6.5 mgm. per 100 cc.) is reached at from four to six months. A steady fall is then evident; however, reasonable constancy exists for the age period three to twelve years (average value slightly in excess of 5.0 mgm. per 100 cc.). The fall to the average adult level (approximately 3.4 mgm. per 100 cc.) apparently takes place rather abruptly between the ages 16 and 20 years.

The clinical conditions wherein significant deficits of serum inorganic phosphate are encountered include rickets, osteomalacia, and hyperparathyroidism. Values above normal are observed in terminal nephritis, following major fractures in adults, hypoparathyroidism and following excessive administration of vitamin D as cod liver oil or viosterol.

Phosphatase. Credit is due in particular to Robison, and to Kay (1932) for recognizing the presence of phosphatase in the blood serum (or plasma), working out a method for its estimation and recognizing its rôle in intermediary phosphorus metabolism, in particular in connection with calcification. Although the method for phosphatase developed by Kay (1930) has been widely used, the modification introduced by A. Bodansky would appear to put the estimation on a sounder basis. According to the method of Bodansky (1933), a unit of phosphatase activity is defined as "equivalent to the actual or calculated liberation of 1 mgm. of phosphorus as the phosphate ion during the first hour of incubation at 37°C. and pH 8.6, with the substrate containing sodium beta-glycerophosphate, hydrolysis not exceeding 10 per cent of the substrate." With this method the range of normal values for plasma or serum phosphatase activity in adults is 1.5 to 4.0 units and in children 5.0 to 13.0 units per 100 cc. Greene, Shattuck and Kaplowitz (1934) obtained a mean of 6.3 units with a standard deviation of 2.2 for their control cases, and regard values between 2.0 and 11.0 as without definite pathological significance. In rickets Bodansky and Jaffe (1934b) found the serum phosphatase activity was 20 to 30 units in mild cases, as high as 60 units in severe cases and 60 up to 190 units in very severe cases. Plasma phosphatase activity may be regarded as reliable means of detecting latent and active rickets and may be em-

ployed as a prognostic index in this condition. Increased serum phosphatase activity is generally present in osteitis deformans (Paget's disease), in which several bones are involved, the values in some cases being very high. Moderately increased values have been found to occur in hyperparathyroidism. Increased phosphatase activity has been observed in both obstructive and hepatogenic jaundice, but elevated values are about twice as common in the former as in the latter.

Sulfate. The normal range of inorganic sulfate appears to be from 0.3 to 1.0 mEq. of S per liter (Denis, 1921; Wakefield, 1929; Hoffman and Cardon, 1935; Letonoff and Reinhold, 1936). Attention has been given chiefly to the changes of serum inorganic sulfate in renal diseases. W. Denis in 1921 noted elevated values (to 10 mEq. per liter) in uremia and subsequent work (Wakefield, 1929; Loeb and Benedict, 1927; Wakefield, Power and Keith, 1931; Hoffman and Mansfield, 1936; Wakefield, Power, and Keith, 1939) has shown that sulfate is retained in the blood when renal function is greatly impaired. The elevations of serum inorganic sulfate appear to parallel non-protein nitrogen retention and Wakefield et al. (1939) recorded a value as high as 20 mEq. per liter. Wakefield, Power and Keith (1931) reported that fairly frequently patients with hypertension or with early glomerular nephritis showed some elevations of the serum inorganic sulfate while common tests for renal function showed no abnormality. Consequently the determination of inorganic sulfate was suggested as an early test of renal function. These findings are not in accord with those of Hoffman and Mansfield (1936) who observed that in renal disease the serum inorganic sulfate increased roughly parallel with the blood urea and from all indications could not be used as a test for early renal inefficiency. Recently Wakefield, Power and Keith (1939) have tempered their original conclusions and state "that the concentration of serum sulfate may exceed the normal limits in cases of mild, rather than early, renal insufficiency." With no apparent explanation, some elevations of serum inorganic sulfate have been encountered in conditions other than renal disease (Wakefield, 1929).

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METALLIC ELEMENTS AND BLOOD FORMATION

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For the purpose of this review the term "blood formation" is limited to the formation of red blood cells and of hemoglobin. During recent years several reviews have been published which cover certain phases of this subject more extensively and from a different viewpoint than this paper (1–19).

I. FORMATION OF RED BLOOD CELLS AND HEMOGLOBIN. *Histogenesis.* In the early stages of embryonic life the liver and the spleen are the main centers of erythrogenesis (2, 15, 422). Later, the bonemarrow assumes this function and in extrauterine life it is normally the main or sole site for the formation of red cells. In periods of stress, however, other organs, particularly the spleen (154, 280) may contain extensive erythropoietic foci. The size of the erythrogenic organ is variable, in early life all of the marrow cavities being filled with active red marrow which is later partly replaced by fatty marrow. Under the influence of hematopoietic stimuli the red marrow can enlarge at the expense of fatty marrow and remain in a state of hyperplasia for long periods (14, 134, 280, 292).

Most hematologists seem to regard the circulatory system of the bonemarrow as a closed bed within which the red blood cells are formed and retained normally for extensive maturation (15, 94, 95, 102, 319). The endothelial cells of the intersinusoidal capillaries are the units from which the red cells ultimately (15, 96, 425) originate. It is generally agreed that histogenesis of the erythrocyte proceeds from the small endothelial cell through the large megaloblast and the more or less differentiated erythroblasts to the normoblast (9, 15, 96, 258, 425). During this maturation the large nucleus of the megaloblast shrinks and in mammals it is extruded or disintegrates as the normoblast matures into a reticulocyte. The reticulocyte is normally the most immature form of red cell entering the circulation, where it matures rapidly into an erythrocyte (169, 306).

Chemically, the stroma of the erythrocyte has not been extensively

studied to determine if factors necessary for the development of the erythrocyte are integral parts of the stroma.

Hemoglobin. Hemoglobin is a protoheme-globin compound. For the purpose of discussion and as a guide in studies on its formation and metabolism hemoglobin can be considered as consisting of globin, protoporphyrin IX and iron. (The protoporphyrin obtainable from hemoglobin is designated as protoporphyrin IX, belonging to the etioporphyrin III series, according to Hans Fischer's system of nomenclature (121).) Although all hemoglobins of different species have the same fundamental property of dissociable combination with oxygen, they exhibit sufficient differences in composition and in chemical and physical properties (420) to permit the conclusion that there are different hemoglobins. Thus the sulfur and cystine content of the hemoglobins of different species show significant variations (51). Measurements of the resistence to denaturation by dilute alkali indicate the multiple nature of hemoglobins of different animals (176) and, more surprisingly, the occurrence of different hemoglobins in the maternal and fetal blood of man (48, 49, 62, 162, 163, 214, 388). The same conclusion is reached from the shift to the left of the oxygen dissociation curve of fetal blood (25, 152, 163). Shortly after birth the fetal hemoglobin begins to be replaced by that normally occurring in adult blood (62) but even in the adult the hemoglobin may not be homogeneous (62, 63). The globin moiety of the hemoglobin molecules seems to be responsible for these differences.

As far as is known the naturally occurring hematin compounds belong structurally to the etioporphyrin III series. This applies to the hemoglobins of all higher animals investigated including the chicken and the pigeon (299, 314), to myoglobin (314, 342), to catalase from liver (364), to free intracellular hematin (198), and probably also to peroxidase (199, 216). The nature of the hematins of the cytochromes is not yet clearly established. Such a striking identity in one of the components of the different hemoglobin molecules and of other hematin proteins not only points to the fundamental physiological importance of this molecular configuration but also suggests that its synthesis in each case involves the same chemical reactions.

Site of formation of hemoglobin. Studies on the histogenesis of red cells indicate a gradual accumulation of hemoglobin during the development of the cell (143). The amount of hemoglobin in the megaloblast is very small, obscured in ordinary staining procedures by basophilic constituents of the cell (9, 15). As the cell matures the basophilia

gradually gives way to the acidophilic hemoglobin. The seat of hemoglobin synthesis, at least in its final stages, is apparently in the nucleus of the developing cell (206, 400, 424). By histochemical reactions iron has been detected in the nuclei of the developing red blood cell (238, 281, 308) and this iron is apparently incorporated into the hemoglobin molecule. Thus the final synthesis of hemoglobin seems to be localized in the erythrogenic tissue within the developing blood cell. The rapid destruction of extracellular hemoglobin and the impermeability of the cell membrane to hemoglobin lend further support to such a theory (104). Whether myoglobin and other hematin compounds are similarly elaborated in situ is not known. The close relation between the formation of red cells and of hemoglobin also raises the question: Can erythrocytes mature without at least a partial complement of hemogobin? If such is not the case the factors necessary for the formation of red cells cannot be as sharply separated as many authors have implied.

II. Synthesis of hemoglobin and its component parts. *Protoporphyrin.* Improvement in methods of analysis and identification of porphyrins have led to a gradual discard of the idea (see 333) that protoporphyrin is a physiological product of degradation of hemoglobin. Recent studies on the formation of bile pigments from hemoglobin do not indicate that protoporphyrin is an intermediate in this reaction (32, 220). Much evidence has accumulated, however, which supports the view that protoporphyrin is an intermediate in the synthesis of hemoglobin. Protoporphyrin is normally present in the erythrocytes (120, 138, 317, 397, 399) and particularly in the reticulocytes (69, 344, 345, 406) but not in blood plasma (218, 399). In fact Watson has suggested that the supravital staining of reticulocytes with brilliant cresyl blue leads to precipitation of the dye and of protoporphyrin (406). The fluorescence of young red blood cells (272, 343) may be caused by protoporphyrin. Protoporphyrin has been identified in megaloblasts and in erythroblasts of fetal bonemarrow (55, 210). Langen and Grotepass have reported an increased protoporphyrin content of the bonemarrow and of the red blood cells following hemorrhagic anemia of rabbits (218). The developing chick embryo contains protoporphyrin in relatively large amounts (55, 137, 398). Grotepass has identified the protoporphyrin of the erythrocytes as protoporphyrin IX (138). Thus, an experimental basis is provided for the theory (18, 55, 104, 166, 196, 220) that protoporphyrin accumulates at the site of hemoglobin formation as an intermediate. Further support of this theory comes from a study of porphyrin excretion in various blood

dyscrasias and in experimental hemorrhagic anemia of dogs (98, 99, 100, 101). Accordingly Dobriner and Rhoads postulate that excretion of coproporphyrin I is proportional to and an index of the formation of protoporphyrin, and hence of hematopoietic activity (100). Hans Fischer's dualistic theory of porphyrin synthesis receives thus experimental support. Rimington has recently advanced an "enzymic theory of hemopoiesis" in which he suggests that coproporphyrin may be a by-product of protoporphyrin synthesis (311). The normal quantitative predominance of protoporphyrin IX synthesis is conditioned by a "directive control" of the "porphyrinosynthetic enzyme system." While admittedly speculative, Rimington's theory may be worthy of experimental approach.

Dietary precursors of pyrrole compounds or of protoporphyrin are not known. Nobody has yet succeeded in producing an anemia due to a deficiency of porphyrin precursors. Experimental hemorrhagic anemia of dogs (312) and nutritional anemia of rats (209) failed to respond to therapy with pyrrole derivatives although iron was supplied in the latter case. Reports to the contrary (182, 295, 296) can at best be interpreted as suggesting that the animal may utilize preformed pyrrole or porphyrin compounds for porphyrin synthesis. As far as is known the animal does not depend upon a dietary supply of pyrrole or its derivatives (18).

Iron. The bonemarrow contains considerable quantities of iron which are decreased in hemorrhagic anemia (53, 124, 147) and in nutritional anemia (339). Some of the iron of the bonemarrow is non-hematin iron (339), perhaps combined with nucleoprotein (278) or lecithin (133). Upon administration of iron its accumulation in the bonemarrow has been demonstrated (53, 301, 339).

Globin. Clinically, a clearcut anemia due to globin deficiency has not been described (104). Bethell has reported increased serum albumin and improvement of anemia of pregnancy following increased milk intake (42). In patients with hypochromic or pernicious anemia Heath and Taylor (171) observed a good response to specific therapy in spite of a very low protein intake. Plasma and tissue proteins apparently served as the source of protein for hemoglobin formation. Experimentally, retarded recovery from anemia (149, 297) as a result of quantitatively or qualitatively inadequate protein intake can be demonstrated readily. Diets very low in protein can produce a macrocytic anemia and megaloblastic bonemarrow in pregnant rats (40, 41, 42, 217). This is probably not the result of an uncomplicated globin deficiency, however.

In view of the remarkable specificity of the globin molecules it might be suggested that they are synthesized in the bonemarrow (or in the muscle cells in the case of myoglobin) from the constituent amino acids. Whipple and his associates on the other hand have repeatedly expressed the opinion that the "liver is active in producing intermediates which go into the manufacture of the large hemoglobin molecule" (147, 313, 375) and that the liver can "assemble the protein-building materials which form the globin fraction" (165). Further work is needed to clarify this point.

Assuming then on the basis of the past discussion that the hematopoietic centers elaborate protoporphyrin IX and globin, and that nonhematin iron is available, the final step of the synthesis of the respiratory pigments may take place in the following manner:

1. Protoporphyrin combines with iron to form protohematin (198) which, after reduction to protoheme readily combines with globin to form hemoglobin (179, 314).

2. A globin-protoporphyrin compound is formed (178, 180, 181) which, upon incorporation of ferrous iron, yields hemoglobin.

III. Methods for the study of the relation of metals to blood formation. The normal organism, with some exceptions, is not well suited for studies of the problem under consideration. In general, conditions under which blood formation is either retarded or accelerated provide the best opportunity for rational study. Clinically this is realized in the various forms of anemia. Some similar conditions are encountered naturally in animals (23, 56, 78, 153, 257, 277, 349, 395). In both cases, however, the abnormal blood picture may be caused by several factors and conclusions from therapeutic measures need cautious interpretations. The action of metals in blood formation is best studied in animal experiments where the experimental conditions particularly with respect to body storage and dietary intake of various factors can be controlled. Except in the production of polycythemia the primary hematopoietic stimulus seems to be low oxygen tension in the tissues. In any form of anemia this condition is apparently realized. Some investigators (211, 232) have used low atmospheric oxygen tension to stimulate bonemarrow activity; others have used acute hemorrhagic (156, 186, 411; 418, 419) or hemolytic anemia (156, 207, 275). In such experiments bodily stores of metallic elements are likely to complicate the results as illustrated by the failure of dogs to respond to iron therapy (411, 418). Chronic hemorrhagic anemia of dogs and nutritional anemia of rats and other species appear to provide the best material for studying blood formation. Experiments with purified diets have thus far not

been very successful because it is difficult to prepare a purified diet that is both adequate and sufficiently low in metallic elements. Recent progress in the preparation of pure vitamins should make this experimental approach feasible in the near future.

Whipple and his associates have developed to a high degree of perfection the technique of producing chronic hemorrhagic anemia in dogs for the study of blood formation (413). Although his standard rations are adequate to maintain mature dogs in a normal state of health for long periods they are partially deficient in more than one factor affecting hemoglobin regeneration. They are low in proteins (413), riboflavin (141) and iron (413) and a uniform intake of copper is not provided (159). The interpretation of the results obtained with this method is sometimes difficult because the necessity of restricting the diet to only one factor affecting blood formation has not been clearly recognized in many of the experiments. Thus it would be interesting to compare the results of liver feeding with those obtained when equivalent amounts of iron, copper, riboflavin and purified protein are supplied. The effect of vitamin B_6 on blood formation also deserves close attention (126). Whipple's technique has been applied successfully in some other laboratories (44, 245, 269, 302, 307, 368) and the need for more adequate control of the diet has been stressed (302, 307, 368).

Nutritional anemia of rats has been used most extensively in the work of Hart and Elvehjem and their associates. With proper precautions this technique is highly successful and it has been used in many laboratories mainly for studies on the effect of iron and copper on blood formation. The basal diet of milk proves particularly satisfactory because it has such a low content of iron and copper and because it supports excellent growth to maturity when fortified with iron, copper and manganese (203). As far as is known, such milk contains all dietary factors necessary for very rapid recovery from nutritional anemia and for maintenance of a normal blood picture. The statement that the "value (of milk) for manufacture of hemoglobin and red cells is but slight" (14) applies only to its limitations in iron and copper content. Nutritional anemia on milk diets is of the hypochromic type (122, 153, 338). Foster (125) and Fitz-Hugh et al. (122) reported microcytosis in rats, while Hamilton et al. (153) observed a normal corpuscular volume in pigs suffering from milk anemia. The scarcity of detailed hematological studies on an anemia which can be so well controlled is rather surprising.

Recent improvements in the field of bonemarrow culture open a new approach to the study of the function of metals in blood formation (52, 289, 290, 291, 407).

IV. THE RELATION OF IRON TO BLOOD FORMATION. As a component of the hemoglobin molecule, iron occupies a key position among the factors affecting blood formation. The best iron analyses of hemoglobins of various species have yielded uniform values of 0.335 per cent Fe (70, 187, 267, 298, 427). The same figure has been given for myglobin (376).

Pre-natal iron stores and iron-metabolism in infancy. Most mammals at birth are stocked with a considerable iron reserve. In addition, the blood of infants and some animals has an abnormally high hemoglobin content. During pregnancy relatively large amounts of iron are transferred across the placenta (331). Iron deficiency of the mother does not necessarily result in lower hemoglobin of the young at birth (22, 367) but it does decrease the fetal iron stores and hasten the onset of anemia during the nursing period (22, 294, 367, 387). Pigs are particularly susceptible to early anemia of iron deficiency (153, 231, 339). The iron content of fetal liver is relatively high (129, 184, 303, 426), reaching a maximum at term. Bunge pointed out fifty years ago that the mineral composition of the fetus corresponds closely to that of milk except in iron and he warned against the exclusive use of a milk diet through extended nursing periods (67, 68). Later work has confirmed this view and has indicated that the iron concentration in the tissues, particularly in the liver, decreases during the nursing period (231, 303, 355, 378) even though the absolute amount of iron may increase. Lintzel and Radeff (231) have reviewed the pertinent literature.

Destruction of hemoglobin *in vivo* does not necessarily lead to extensive loss of the iron liberated. Although metabolism studies in earliest infancy have shown a pronounced negative iron balance (37, 192, 361, 362), the iron requirement of the growing infant can be satisfied, for a short time at least, from the iron reserves in the liver and also from the physiological destruction of excess hemoglobin (192, 361, 362). Similar conservation of iron liberated during excessive hemoglobin destruction has been observed in cases of polycythemia vera treated with phenylhydrazine or its derivatives (34, 207, 248, 305). Likewise in the formation of bile pigment from injected hemoglobin or myoglobin the liberated iron can be stored in the tissues or, if necessary, utilized for the formation of new hemoglobin (150, 164, 242, 259, 279, 409).

Tissue and storage iron. The iron content of animal tissues, particularly of the liver, is subject to marked variations depending on the balance between absorption of dietary iron, excretion of iron and the requirement for hemoglobin formation. The chemical nature of the iron

compounds in the tissues is not clear. Varying fractions of the total iron (generally between 15 and 70 per cent) are soluble in acetate buffer or dilute trichloracetic acid and react with $\alpha\alpha$-bipyridine upon reduction. This iron is nonhematin iron and probably includes such compounds as "ferretin" (219) and dissociable ferric protein complexes (76, 119, 250, 356). The association of ferric iron with phosphorus compounds has been suggested (119, 133, 250, 278, 385). Another fraction of tissue iron can be ascribed definitely to hematin compounds. Small amounts of inorganic ferrous iron may also occur in tissues. These different iron compounds show variations in the ease with which they are mobilized to become available for hemoglobin formation. A mobilization of tissue iron as a result of rapid hemoglobin formation has been demonstrated frequently (113, 417, 419). The liver and spleen of even the most severely anemic animals contain considerable amounts of iron however (83, 113, 147, 173, 339) partly as non-hematin iron (148, 339). These iron compounds are not withdrawn from the tissues for hemoglobin formation (147, 173, 192, 193, 339) even when all other factors for rapid hemoglobin formation are available (339). The heart and the skeletal muscles are particularly resistant to iron depletion. Severe anemia fails to reduce the myoglobin content of skeletal and heart muscles (6, 81). In the heart it can be even increased due to hypertrophy of the organ (81). A clear distinction between mobile and immobile iron has therefore been made only on a physiological basis.

Dietary iron in relation to hemoglobin formation. For maintenance of the normal rate of hemoglobin formation and for preservation of tissue iron the organism depends on an adequate dietary supply of iron. For a discussion of the extensive literature on absorption, excretion and dietary requirements of iron the reviews on iron metabolism and the more comprehensive papers on the subject should be consulted (6, 7, 11, 16, 27, 65, 247, 270, 271). McCance and Widdowson have introduced the idea that the intestine controls the amount of iron absorbed (247, 249, 415). Recent work from Whipple's laboratory with radioactive iron has strengthened this view and has indicated that the absorption of iron from the intestine is affected or controlled by the needs of the body (144, 145). The factors operative in such a control are entirely obscure. High iron retentions, however, have been observed in normal and anemic subjects (65, 127, 128). The various effects of other dietary factors on iron absorption that have been recorded in the literature do not permit a correlated rational interpretation. Thus increased alkalinity seems to depress iron absorption and the therapeutic

effect of iron salts (202, 254) while acidity is favorable. High calcium intake or a high Ca/P ratio interferes with iron utilization according to some authors (205, 347), while others observed the development of anemia on a reduced calcium intake (89). The dissociation of iron salts in the intestinal tract has a marked effect on solubility and the ease of absorption of iron. The clinical use of iron pyrophosphate is of special interest in this connection (109, 282, 332).

The therapeutic ineffectiveness of small doses of iron apparently cannot be satisfactorily explained on the basis of poor absorption (64, 127, 304). Large doses of iron compounds have often been effective in the clinical treatment of iron deficiency anemia where smaller doses failed (28, 255), and their use is generally recommended (64, 127, 366, 423, 424). The utilization of the administered iron in terms of hemoglobin formed is generally quite low (127) and not directly proportional to the dose of iron. Animal experiments have yielded similar results (36, 302, 414).

In contrast, iron injected intravenously or parenterally has been recovered almost quantitatively as hemoglobin iron (164, 170, 173, 414). Further work will probably show whether the lower effectiveness of iron administered orally in equivalent amounts can be explained solely on the basis of incomplete absorption.

Clinical experience in the treatment of anemia has often indicated a greater efficacy of ferrous than of ferric salts (88, 237, 352, 423, 424). Some animal experiments have shown similar results (79, 105) while in others no difference could be observed (147, 390, 412). Better absorption from the intestine is supposed to account for the superiority of ferrous salts (79, 105, 264). The work of Lintzel (230) has shown that $\alpha\alpha$-bipyridine inhibits the absorption of iron from the intestine due to the formation of the Fe^{++} bipyridine complex. It has been mentioned previously that ferric salts have a marked tendency to form complexes with proteins and phosphorus compounds. Their absorption may thus be affected. Ferrous salts, in contrast, are less susceptible to complex formation (16). If it is true that only ferrous salts can cross the intestinal barrier, the factors affecting reduction of ferric salts in the intestine would have a deciding influence on the absorption of iron and might explain the discordant results of different investigators. This problem deserves closer study.

In cases where intravenous iron therapy is desirable, the use of ferrous adenylate (318) and ferrous ascorbate seems promising. The latter compound has been used by several investigators (123, 130, 132, 172,

350, 365) with good results. Its use deserves further investigation. It may also be mentioned that ferromagnetic iron (408) has no special hematopoietic value (97, 401) as compared with other iron compounds.

During recent years the "availability" of iron compounds, particularly of the iron in natural food materials, has received considerable attention. Based on the observation that the iron of orally administered hemoglobin or hematin was not utilized by anemic animals (73, 108, 177, 229, 283, 348), Elvehjem and associates found that the utilization of the iron in natural food materials by rats under standard conditions was not correlated with the total iron content, but corresponded to the non-hematin, "bipyridine iron." The hematin molecule apparently cannot be absorbed, or, if absorbed, its iron is not liberated (103). Injected hemoglobin or myoglobin is well utilized for hemoglobin formation (164, 375, 410). Iron of the hematin-proteins is readily split off in the formation of bile pigment while the uncombined hematin molecule is more resistant to disintegration (103). The theory of Bunge that only food iron was absorbed and utilized (66, 256) has long been discarded in view of the clinical effectiveness of inorganic iron salts, and now the new concept of "available iron" has provided a more rational basis for the evaluation of foods as sources of iron for the organism. The agreement between chemical and bioassays for "available iron" in general has been satisfactory (348, 353, 354) although some discrepancies have been noted (24). The effect of other constituents may influence the absorption of the iron of the food material assayed. Modifications in the chemical (54, 208, 346, 380) and bioassay methods (157, 353, 354) may produce closer agreement of the values obtained. Further work is also necessary to show whether the results can be extended to species other than the rat (282). The criticism (148) that the term "available iron" has no physiological significance is due to misinterpretation, as Sturgis et al. (17) have pointed out. Studies on "available iron" have received clinical attention (194, 282).

Blood iron and iron transport. The predominance of hemoglobin iron in blood is so great that the difference between total blood iron and that calculated from the hemoglobin content is small enough to fall within the limits of error of many older analytical methods. Many studies during the last 15 years have definitely established, however, that blood contains at least two other iron fractions besides hemoglobin: *a*, serum or plasma iron, and *b*, "easily split-off iron" (leicht abspaltbares Bluteisen) in the red blood cells. Normal human blood serum contains about 100 micrograms of iron per 100 cc. of serum of

females and about 120 micrograms per 100 cc. of serum of males (173, 263). The "easily split-off iron" of the red cells constitutes from 5 to 10 per cent of the total iron (30, 263). Until recently the chemical nature of the latter fraction was uncertain although it was recognized early (30) that it was not associated with porphyrin. Barkan finally succeeded in identifying it as a component of the pseudohemoglobins, (the verdohemochromogens of Lemberg (221)), nonporphyrin intermediates in the breakdown of hemoglobin to bile pigment (32). The earlier view that it was transport iron (30) has been dropped. Instead, more recent studies have definitely associated the iron of the plasma with iron transport (173, 265).

The following picture of iron transport seems to be valid on the basis of available information. Iron absorbed in the intestine, presumably as ferrous iron, passes directly into the bloodstream (not through the lymph) (264). The erythrocytes are apparently impermeable to iron (31, 360) added *in vitro*. If this is true *in vivo* it would indicate that absorbed iron is incorporated into the maturing blood cells of the bonemarrow very rapidly because Hahn et al. have presented evidence that radioactive iron appears in the red blood cells a few hours after feeding it to anemic dogs (145). In the plasma the ferrous iron is oxidized to ferric iron (31, 175, 360) and carried in non-dializable, complex form (29, 359). With the improved analytical methods marked variations in plasma iron have been detected following iron feeding and in some blood dyscrasias. Thus feeding of ferrous iron causes increased values for serum iron which are more marked than those obtained after feeding of ferric salts (173, 264). The highest concentration of serum iron is reached in two to five hours after feeding (46, 145, 265). In early infancy, serum iron is greatly increased (195, 377) while iron deficiency anemias are characterized by low serum iron (173, 265, 309, 310, 402). Clinical states of diminished hemoglobin formation not due to iron deficiency present high values for serum iron (155, 174, 310, 402) which diminish during recovery. Moore et al. (265) have pointed out that plasma iron "is influenced by and a measure of *a*, the amount of iron absorbed from the gastrointestinal tract; *b*, the extent and adequacy of the iron reserves of the body; *c*, the ability of the bonemarrow to utilize iron in the synthesis of hemoglobin; *d*, the rate of hemoglobin synthesis; *e*, the extent of hemolysis taking place in the liver and the spleen."

Mechanism of the action of iron. The function of iron as a constituent of hemoglobin is of course evident. But since the formation of hemo-

globin and of erythrocytes are interdependent, to some degree correlated processes, iron also affects erythropoiesis. The development of the red cells in the closed intersinusoidal capillaries takes place in a medium of low oxygen tension (15). The immature forms of the red blood cells have a measurable respiratory activity (85, 93, 158, 185, 233, 266). The intervention of enzymes of the hematin type in the respiration of these cells is very probable and hence iron would be necessary for the development of the immature red cells. In this connection the question arises whether respiration and glycolysis of immature red blood cells are necessary to supply energy for the synthesis of hemoglobin within the cells. In iron deficiency anemia erythropoiesis is supposedly arrested in the normoblastic stage (143, 425). But since even severe anemia does not totally deplete the iron reserves, an effect of iron on earlier forms of cell development cannot be denied. Decreased porphyrin excretion in iron deficiency has been interpreted to indicate that iron is instrumental in the preporphyrin stage of pigment synthesis (104). Heilmeyer and Plöttner have reported that intravenous injections of ferrous ascorbate lead to hemoglobin synthesis in excess of that calculated from complete utilization of the iron supplied (173). If this is true in cases of uncomplicated iron deficiency, a stimulating action on the bonemarrow and subsequent mobilization of iron reserves must occur. The view that iron has a stimulating effect on erythropoiesis has frequently been expressed (112, 142, 170, 189). To what extent the later stages of cellular differentiation are affected by hemoglobin synthesis remains to be determined.

V. COPPER IN RELATION TO BLOOD FORMATION. *In animals.* During their studies on nutritional anemia produced by exclusive feeding of milk, Hart and his associates observed that pure iron salts failed to exert a curative effect unless other supplements were fed (see 5). The active principle of these other supplements was identified as copper (161), and it was shown that a rapid and complete recovery is achieved by severely anemic rats through the combined effects or iron and copper therapy. McHargue et al. (253) shortly afterwards published experiments indicating that copper has a marked effect on blood formation of rats. The development of this work, its subsequent confirmation in many laboratories, and the failures reported by some others have been discussed in the review by Elvehjem (5). The best proof for the necessity of copper and the ineffectiveness of pure iron salts lies in the fact that progressively severe and fatal anemia can be produced when the milk diet of young rats is supplemented with adequate doses of pure iron from

the time of weaning (287, 337, 389). The amounts of copper effective in the cure of anemia are exceedingly small; 5 micrograms of copper per day, added to a diet of milk and pure iron suffice for rats to initiate and to maintain a steady formation of hemoglobin and of erythrocytes (338). A more rapid response is obtained with larger amounts of copper.

Studies on the hematopoietic effect of copper have been extended to several species and the results thus far have been uniformly positive where rigid precautions were taken to exhaust the animals of copper reserves and to maintain the intake of copper in the basal diet at a minimum. This applies to chickens (111), sheep (379), swine (110, 339) and dogs (131, 302). Even under practical conditions, localities have been found (277, 349) where cattle, sheep and goats suffer from copper deficiency and where the resulting anemia is not cured by feeding iron alone. The contention by Hahn and Whipple (148) that copper "has not been demonstrated to be lacking in any condition except nutritional anemia in rats and so cannot be considered in any way as a limiting factor in the anemia of dogs due to blood loss" seems inconsistent with the results reported from their own laboratory (107) and with the experiences of others (131, 302).

In man. The demonstration that copper is essential for blood formation in animals led to its clinical trial in various forms of anemia. The results have not been uniform, as might be expected in conditions of varying etiology involving small amounts of an active principle that is widely distributed in nature. The experience with animal experimentation has emphasized the importance of tissue storage and of previous dietary history in the study of trace elements. These considerations can provide an explanation for the frequent effectiveness of iron, without added copper, in the treatment of anemias of iron deficiency in man. Several investigators have reported that addition of copper to iron medication did not improve the results obtained with iron alone (1, 43, 88, 168, 202, 237, 240). Others have found only isolated cases in which failure of iron therapy could be corrected by copper salts (86, 87, 293).

Some of the literature on the effect of copper on blood formation in humans has been reviewed by Hutchison (183). In his own studies he fed iron to anemic children for one to three weeks. When iron therapy was discontinued and the hemoglobin had reached a constant level, copper was fed, with the result that stored iron was mobilized and utilized for further hemoglobin formation. Lewis (227) reported observa-

tions on 34 anemic children who failed to respond to iron therapy but who were cured by subsequent copper and iron therapy. Usher et al. (396) observed a large group of infants during a prophylactic experiment in which the results of iron medication were compared with those of combined iron and copper treatment. The effect of copper on the production and maintenance of a higher hemoglobin level was striking. From 1 to 2 mgm. of added Cu per day was sufficient in this and in other studies (109, 183) to supplement iron therapy. Many other cases substantiate the superior effect of copper and iron therapy in the cure of nutritional anemia in children, (71, 72, 189, 191, 213, 223) often achieved after failure of therapy with iron alone (109, 167, 293).

The effect of copper on blood formation in anemia of adults, particularly in chronic idiopathic anemia, has also been demonstrated. Mills (260, 261) observed 31 cases of chronic idiopathic anemia, refractory to iron, which responded to copper therapy. The experience of Adamson and Smith (21) is similar. In view of these and other results (86, 87, 188, 239, 334) it is evident that copper is necessary for the formation of hemoglobin and of erythrocytes in man as well as in animals. There is no evidence to date that the mineral requirements for synthesis of hemoglobin and for the formation of erythrocytes are qualitatively different in those species investigated thus far. The broad statement by Hahn (6) with regard to copper that "*from a practical standpoint there is no indication whatsoever for the inclusion of this element in anemia therapy*" (italics are his) hardly agrees with the facts published in the literature. Acute copper deficiency in man, particularly in adults, is perhaps not very common. No satisfactory analytical or diagnostic procedure is available for its detection to date, and its clinical recognition still depends on the success or failure of empirical treatment.

Copper storage in tissues. The liver of the young is characterized by a relatively high copper content at birth and provides a significant reserve which is drawn upon while the copper intake is low during the nursing period (38, 83, 146, 224, 225, 252, 268, 303). A high copper intake of the mother does not raise the copper content of the young appreciably (160, 228) but copper deficiency of the mother may decrease the copper reserves of the young (222). The copper content of cow's milk is low, about 0.10 mgm. per liter (75); that of human milk, particularly of colostrum, is probably somewhat higher (135, 226, 262). During the development of anemia on a milk diet, the copper reserves of the tissues are gradually depleted to very low levels (47, 222, 339). Feeding of copper can restore the reserves and lead to a great accumulation of copper in the liver (58, 80).

During embryonic development copper becomes concentrated in the liver (236, 251, 303, 369, 416) which is significant in view of the hematopoietic activity of embryonic liver. In the human fetus the copper concentration reaches a maximum at term (303) while in the embryos of chickens (251) and pigs (416) the highest percent copper is attained at an earlier stage.

Data on the copper content of the bonemarrow are few. Analytical limitations and the difficulty of obtaining satisfactory samples from small animals have been a serious handicap. Sarata (329) reported a copper content of about 3 mgm. per kgm. of red marrow and about 0.8 mgm. for fatty marrow of rabbits. He stated that after repeated bleedings the copper content of the red marrow increased. Analyses of human red marrow (38) and of the distal ends ribs (339) of pigs fall into the same range. Cunningham reported a higher copper content for the red marrow of a calf (83). More information on this point is needed particularly with respect tc possible variations during different states of hematopoietic activity (339).

Form of copper in tissues. In contrast to iron, copper of tissues and of blood can be completely extracted with trichloracetic acid (250, 382, 384). It is apparently loosely combined with protein (405). Gruzewska and Roussel (139) have isolated an albumin fraction from calves' liver containing 0.21 per cent copper. Hemocuprein and hepatocuprein, isolated by Mann and Keilin contained 0.34 per cent copper (241). In the blood, copper occurs in a non-dializable form (20, 57), probably combined with albumin (106). Upon addition of acid blood copper becomes dializable (57). Tompsett has suggested that copper in the erythrocytes may occur in the cuprous form, combined with glutathione (381, 382).

Copper in the blood and its variations. An extensive literature on the copper content of the blood has accumulated in recent years. The values for normal blood of different species have been tabulated in several papers (234, 316, 327, 382). Normally the blood of man and of most animals studied contains about 120–150 micrograms of copper per 100 cc. Studies on the distribution of copper between plasma and cells have not given uniform results. Sarata and his associates have consistently found a relatively high concentration of copper in the red blood cells (328, 371). Others report a more equal partition of blood copper (50, 382, 386) or a higher concentration in the plasma (340). The relatively high copper concentration in the avian nucleated red blood cells is quite striking (235, 405).

Clinically, a significant increase in blood-copper during pregnancy

has been observed by several authors (136, 212, 234, 321, 322, 324, 386). In fetal blood, at term, however, the copper concentration is much lower (222, 322, 324). This may be indicative of mobilization of copper in the mother and of storage in the fetus. Hemorrhagic anemia in both the acute (186, 234, 235, 320, 330, 406) and the chronic form (320) leads to an increased copper content of the blood. The same has been observed following phenylhydrazine injections in man (323) and in rabbits (275). Anemia of children (135, 136, 223), chlorosis (326) and pernicious anemia (115, 234, 326) were found to increase the copper content of the blood. In nutritional anemia of pigs due to copper deficiency however, the copper content of the blood decreased to extremely low values (340), and was restored to normal very rapidly upon feeding 2 to 4 mgm. of copper per day. A drop in the copper content of the blood has also been observed in nutritional anemia of dogs (302) and in prolonged anemia of children (223). These changes in the copper content of the blood are undoubtedly directly connected with the hematopoietic activity of the bonemarrow. An increased copper content of the blood signifies mobilization of tissue copper and presupposes adequate copper stores. The absence of a hemoglobin response to iron therapy in pigs with a very low copper concentration of the blood and the rapid response following restoration of the normal copper concentration suggest that a minimal, as yet undetermined copper concentration of the blood is necessary for normal or rapid blood formation (340). Since the restoration of a normal copper content of the blood in these experiments was not accompanied by an appreciable accumulation of copper in the bonemarrow, it appears that the copper content of the blood has a functional significance in relation to blood formation. A rapid increase of blood copper is evidently not the mere result of increased copper absorption and of transport to the tissues. The increased copper concentration in various other forms of anemia lends support to such a view. The hematopoietic effect of blood serum from animals kept under reduced oxygen tension (211) or recovering from hemorrhagic anemia (357) as well as of the transfusion of blood with added copper (325) is of interest in this connection.

The appearace of "cuprocytes" (copper rich cells) in the circulating blood of rabbits during the early stages of recovery from hemorrhagic anemia has been reported (186). The experiments with pigs however, did not permit such a conclusion (340), although the experimental conditions were different.

Mechanism of the effect of copper on blood formation. Copper is not a

constituent of the hemoglobin molecule (114, 140) and it has not been identified, up to the present, as a necessary component of the structural material of the circulating erythrocytes. Much evidence indicates that copper is not necessary for the absorption and storage of iron in tissues (113) but that it facilitates or is essential for the utilization of iron by the blood-forming organs and for the mobilization of iron from the tissues (26, 77, 83, 113, 183, 190, 195, 273, 302, 421). Without the action of sufficient iron and copper no typical reticulocyte response is observed during recovery from nutritional anemia of rats (338). It has been suggested that the production of reticulocytes depends on the presence of newly formed hemoglobin or its precursors in the developing cells (112, 338). If such is the case copper exerts its action in those stages of erythrogenesis preceding the reticulocyte. Histological studies of the bonemarrow under carefully controlled conditions should help to clarify this question. Several authors have suggested that copper is necessary only for the formation of the red blood cells but not for the production of hemoglobin (45, 115, 300, 335, 363). While it is true that a marked increase of erythrocytes can be observed when hemoglobin synthesis is only slow (340) it must be remembered that even the most severely anemic animals contain appreciable amounts of non-hemoglobin iron which may suffice for the maturation of hypochromic erythrocytes. Further work is necessary to permit a definite decision on this point. It appears that an intensive and correlated study of the blood with respect to its content of copper and plasma iron (195) and other chemical and physical changes during anemia and during rapid recovery deserves particular attention. Together with analytical and histological studies of the bonemarrow under the same conditions such an approach offers much promise for the clarification of the complex mechanism of blood formation.

Quite surprising is the observation (47, 339) that anemic rats show a very low copper retention during short periods of copper therapy, even if recovery from anemia is very rapid when iron is fed simultaneously. Only 4 to 5 micrograms of copper were retained daily under such conditions (339). Whether more copper was absorbed and re-excreted is problematical.

The recent identification of several enzymes as copper-protein compounds (84, 200, 201, 215) emphasizes the catalytic activity of copper compounds. Of special interest also is the observation that copper is absolutely necessary for the formation and maintenance of cytochrome oxidase activity of rat tissues (74, 337) and that the bonemarrow in

copper deficiency shows a low cytochrome oxidase activity (336). In anemia of iron deficiency (where copper is fed), the cytochrome oxidase activity of rats' marrow is distinctly increased. Copper deficiency also leads to a diminished intensity of the spectral bands of cytochrome A in rats' tissues (74).

VI. THE RELATION OF COBALT TO BLOOD FORMATION. Waltner and Waltner (404) reported ten years ago that feeding cobalt caused a polycythemia in rats. Other investigators have confirmed this observation not only with rats (39, 60, 197, 243, 274, 287, 358) but also with dogs (61, 90, 91, 131, 244), pigs (358), rabbits (33, 197, 204), guinea pigs, mice and frogs (370) and with chickens (341). When the diet is copper deficient, cobalt polycythemia is not produced and rats die from anemia (287). The increased erythrocyte counts and hemoglobin values represent a true polycythemia not due to a decreased blood volume (286). Feeding of cobalt to animals on a diet otherwise adequate for blood formation initiates a reticulocyte response (91, 204, 243, 284). Feeding of liver (91), injection of liver extract (243) or of ascorbic acid (33) tend to counteract or nullify the effect of cobalt.

The nature of the cobalt polycythemia is not understood. It appears that in the presence of other factors necessary for blood formation cobalt causes a hyperplasia of the bonemarrow (197, 244) followed by reticulocytosis. The suggestion has been made that cobalt inhibits the respiration of immature erythrocytes and causes their discharge into the circulation (33).

The effect of cobalt on blood formation has assumed new significance with the observation that cobalt is essential for the successful nutrition and the prevention of anemia of sheep (56, 78, 117, 118, 394) and of cattle (276). Cobalt deficiency in these species is observed in some areas under practical farm conditions and it is prevented or cured by feeding cobalt salts. The tissues of sheep suffering from cobalt deficiency contained abundant amounts of iron (392). The cobalt requirement is apparently very small, not more than 4 micrograms per day per kilo body weight of sheep. Rats on a milk diet ingest about 6 micrograms cobalt per day per kgm. body weight (393). Only the use of purified diets or of food materials from the afflicted areas will permit a decision whether cobalt is generally essential for normal function of the hematopoietic organs. It is of interest to note that commercial iron salts as used in massive doses for the treatment of anemia contain small but perhaps significant amounts of cobalt (391).

The cases where cobalt has been used clinically for the cure of anemia are too few to permit conclusions (35, 82, 197, 403).

VII. THE EFFECT OF OTHER METALS ON BLOOD FORMATION. *Calcium.* A microcytic polycythemia in rats has been produced by Smith and Swanson and their associates on diets with a very low salt content (351, 372, 373, 374). The condition develops gradually and uniformly, and it is apparently not due to changes in blood volume. Orten et al. (285) studied this condition further and concluded that it is primarily a result of lacking calcium and/or iron. Day and Stein (92) however suggested that the distorted, low Ca/P ratio of such diets is responsible for suppressed absorption of iron. This may account for the hypochromia but the true cause of the polycythemia has not been explained satisfactorily. The effect of calcium and particularly of phosphorus on the absorption of iron deserves close consideration.

Other metals. The claims made for the hematopoietic effect of germanium dioxide have been reviewed and discussed in the review by Robscheit-Robbins (9). Nothing has been published since that time to alter her conclusions that germanium dioxide has no effect on blood formation. The same applies to the reports that manganese, zinc and vanadium affect the reticulocyte response of anemic rats. Elvehjem (5) has discussed and explained the basis of those claims. To date there is no evidence that metallic elements other than iron, copper and cobalt have any direct effect on the formation of erythrocytes and hemoglobin.

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IMMUNITY IN INVERTEBRATES

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The scientific study of the principles and mechanisms of immunity in invertebrate animals is in its infancy. Consequently, the literature dealing with it is meager and widely scattered. The investigators have often had an interest in it only incidental to some other problem. No comprehensive review in English has yet been made, but an excellent review of the literature up to 1922 was made by Cantacuzène (31) and a few other reviews appeared previous to (69, 150) and since that time (43, 101, 127, 128, 151). This review is an attempt to collect and to evaluate the more important of the works in this field.

The scope will be restricted to metazoan invertebrates, not because of any belief that the immunity principles in the protista are essentially different from those in the metazoa, but because the literature on the former has already received more extensive treatment. The scant literature on non-vertebrate Chordata is, however, included for the sake of convenience, since it will probably not be found in most discussions of vertebrate immunology.

A word of caution seems to be necessary against the use of the term "invertebrate immunity" instead of the one used here in the title, since it might be interpreted in the sense of metazoan immunity, or the immunity in vertebrates to the invertebrate parasites. The term "comparative immunology" has been proposed for this field (150) but it is believed that it might be misinterpreted to mean comparative as between immunology of man and of animals.

Natural immunity. In the invertebrates, as in other organisms, natural immunity and susceptibility to microörganisms and their products is a characteristic of the species, and often of the individual. This is exemplified by the failure of *Agregata eberthi*, a coccidian parasite of cuttlefish, to develop in *Portunus puber*, even though it develops well in various other species of the Portunidae (107); by the complete immunity of the bee-moth (*Galleria mellonella*) to many organisms pathogenic for higher animals, such as many strains of the tubercle bacillus, the diphtheria bacillus, various streptococci, *Mycobacterium*

leprae and *Pasteurella pestis* (118, 121, 122, 135); by the natural immunity of silkworms to *Coccobacillus acridiorum* (d'Herelle) which is very pathogenic for ants, crickets and various caterpillars (39), and by the natural immunities to species of human and avian malarial parasites shown respectively by most species of culicine and anopheline mosquitoes. It is impossible, at present, to predict whether any species of invertebrate not already tested with a specific microbe will be naturally susceptible or immune to the latter or its products. However, a certain generalization has been made in respect to the immunity of the invertebrates and the vertebrates to the organisms pathogenic to the vertebrates and invertebrates respectively (1, 16, 118, 120, 121, 189). Thus the microörganisms tested against caterpillars of the bee-moth could be divided into three groups according to the natural immunity shown by the caterpillars to them. The first group to which the caterpillars showed complete immunity contained, for the most part, organisms pathogenic to higher animals, such as *Mycobacterium tuberculosis, Corynebacterium diphtheriae*, streptococci, *Cl. tetani*, *P. testis* and *Trypanosoma brucei*. Group 2, to which the caterpillars showed an incomplete immunity, contained such organisms as *Staphylococcus aureus, Cl. perfringens, Pasteurella aviseptica, Vibrion septique*, and *Vibrio cholerae*. The organisms of group 3, to which there was no immunity even to very small doses, contained organisms less pathogenic or potentially pathogenic to higher animals, such as *Bact. coli, Ps. pyocyaneus, Bact. prodigiosus, B. subtilis, P. proteus* and *B. anthracoides*. Such a general rule is not without its exceptions; for example, caterpillars of *Achraea grisella* are susceptible to piscine strains of tubercle bacilli when kept at room temperature (117) and the caterpillars of the bee-moth are very susceptible to infection with cholera vibrios and dysentery bacilli (134).

A classic example of individual natural immunity is the resistance shown by a few individual silkworms to *Nosema bombycis*, the causative agent of pébrine (154). Thanks to this individual immunity, Pasteur was able to breed resistant stocks of silkworms. These cases of individual immunity are particularly well known among species of insects acting as vectors of disease in man and other vertebrates. Species of avian *Plasmodium* especially have been studied in this respect. When many individuals of the mosquito, *Culex pipiens*, sucked avian blood containing thousands of infective forms of *Plasmodium cathemerium* or *P. relictum* some of them became heavily infected, others lightly infected and the remainder escaped infection entirely, even though they were

given more than one infective meal (86, 88). Selection of progenies from susceptible and insusceptible females gave respectively stocks with increasing and decreasing degrees of susceptibility (87). This character of insusceptibility behaved as a Mendelian dominant (89). Furthermore, indirect evidence gained by statistical studies of the numbers of normal parasites resulting from two spaced, infective meals showed that the degree of susceptibility is an individual character, probably hereditary (89, 90). It has also been shown that the average sizes of the oöcysts of various strains of these species of *Plasmodium* grown in *Culex pipiens* under carefully controlled environmental conditions often differ significantly in different individual mosquitoes (91), which indicates that the rate of growth of the parasite is to some extent conditioned by the constitution of the individual insect. In this natural immunity the barrier seems to be the intestinal wall, since development of the parasite proceeds to the oökinete stage as well in insusceptible mosquitoes as in susceptible ones (86, 90, 142). This is also the case with *Agregata eberthi* in *Portunus* mentioned above (107). The susceptibility and insusceptibility of a leafhopper, *Cicadella mbila*, to the virus of streak disease of maize has been shown to be hereditary and in this case susceptibility is dominant and sex-linked (170).

The experiments on natural immunity in invertebrates constitute a bare beginning in this field but they have gone far enough to indicate that the mechanisms which determine whether an invertebrate will become infected with a given microörganism are complex and that some of them, at least, are inherent characteristics of the individual, as they are in the case of vertebrates.

Acquired immunity. Although very little is known about the immunity in invertebrates which may be acquired as a result of natural infections, a considerable number of studies has been made upon the experimental production of immunity, or phenomena ordinarily considered as accompaniments of immunity. Attempts to produce immunity or immune phenomena were at first so uniformly negative as to lead to the belief that acquired immunity did not exist in invertebrates. Some of these failures can now be explained by the use of organisms having no pathogenicity for the invertebrate used or by the use of techniques peculiar to mammalian immunology. The many hit-and-miss attempts to produce antibodies in invertebrates by whatever antigens happened to be convenient can be best discussed in the section dealing with the principles of immunity. Let it suffice now to give some of the examples of success and failure to establish experimentally an acquired immunity to various infectious agents. The marine spoon-

worm, *Sipunculus nudus*, (GEPHYREA), has been immunized by four vaccinations at 6 to 10 hour intervals of a vibrio isolated from this species of animal (28). Most of the other successes have been met in attempts to immunize insects. Caterpillars of *Agrotis segetum* (NOCTUIDAE) have been immunized against *Bacillus melolonthae-non-liquefasciens* by inoculating them with a three-month's old culture (146). Twenty-four hours after the vaccination these caterpillars were completely immune to doses of the virulent culture lethal to non-vaccinated controls. Silkworms have been similarly immunized against organisms causing a "Flacherie"-like disease by inoculating them with a killed culture of the organisms (70). Other caterpillars successfully vaccinated against bacteria pathogenic for them are: *Pyrausta nubilalis* (PYRALIDAE) (129), *Galleria mellonella* (42, 94, 95, 120) and *Pieris brassicae* (PIERIDAE) (85). Insects belonging to other orders which have been successfully immunized are: *Carausius morosus* (ORTHOPTERA) (135, 189) and honey-bees (HYMENOPTERA) (136, 176, 177).

There are numerous examples of inability on the part of the invertebrate to develop an immunity to an invader or an antibody to some foreign substance. Insects appear to have poor powers of overcoming parasitic protozoa, fungi (3) and insects (169, 174) once these organisms have invaded their tissues. In many cases there is a complete absence of phagocytosis or other apparent immune phenomena. *Culex* mosquitoes do not acquire an immunity against avian *Plasmodium* through a natural infection of the latter (88, 165). Cockroaches (*Periplaneta orientalis*) and crayfish (*Astacus fluviatilis*) could not be immunized experimentally against *Bact. coli* and *Ps. pyocyaneus* (45). There have been no successes in producing antibodies in echinoderms and annelids, and excepting *Helix pomatia*, none in molluscs.

Most of the authors who have been successful in the production of acquired immunity have remarked about the rapidity of its appearance. In most cases it has appeared to be only a matter of a few hours (132, 134) to a day until it was established (146, 176). The methods used successfully in bringing about the active, acquired immunity are: *a*, injection of microörganisms heated to 60°C. for 30 minutes; *b*, injection of aged cultures; *c*, injection of minimum doses of young cultures, and *d*, the use of bacteriophage. Although some success has been attained in producing immunity by feeding the vaccine to the insects, the method has proved to be inefficient. It has been found that there is an optimum titre for the vaccine beyond which vaccination may prove to be less effective or even fatal to the insect (132, 191).

The specificity of the acquired immunity is open to question. Some

of the earlier studied cases were claimed to be as specific as acquired immunity in mammals (150). The bee-moth has been immunized against the cholera vibrio by the infection of *Bact. coli*, anthrax bacilli, and others and with Chinese ink as well as with the specific vaccine (95). While it was claimed that this non-specific immunity was maintained throughout the life of the caterpillar, there was evidence that vaccinations by the specific vaccine yielded the stronger and more stable immunity. However, in one instance, caterpillars immunized by means of heated cultures of *Micrococcus galleriae no. 3* and *Bact. galleriae* exhibited a stronger immunity against cholera than those vaccinated with the specific microbe (95). Extension of the work on the bee-moth to include such antigens as bouillon, horse serum, and a variety of other bacteria and the use of test organisms other than cholera vibrios has yielded similar results and has, in addition, shown the absence of specificity *in vitro* as well as *in vivo* (41, 188). That the bee-moth is not unique in the production of this non-specific immunity is shown by similar success in immunizing *Carausius morosus* (ORTHOPTERA) (190). In all of these latter cases, however, the specific immunity lasts longer than the non-specific, and in one case, at least, the former lasts still longer following repeated vaccinations of the specific organism (190). It seems reasonable to conjecture that at least some of the precocity in the appearance of acquired immunity may be due to a very rapid non-specific response to the vaccine.

A few attempts have been made to transfer passively the immunity acquired in one bee-moth caterpillar to a non-immune caterpillar (42, 183, 184, 185, 186). By transferring blood from the former to the latter it has been claimed that an immunity against the Danysz bacillus has been acquired within three hours (183). By separating cells from plasma of the blood of the immune caterpillar it was possible to confer passive immunity by either separately (185). Immunity could not be conferred to the same degree by the inoculation of blood from non-vaccinated caterpillars as from vaccinated ones. The question of specificity of this passive immunity is also still open. While there is some evidence of specificity, it seems necessary to point out that in one case there was better protection in the bee-moth against *Coccobacillus acridiorum* by transfer of blood from caterpillars vaccinated with heated Danysz bacilli than from those vaccinated with the former organisms (184), and that the so-called passive immunity is of very short duration. Results comparable to the cases cited above have been obtained by injecting into normal caterpillars of *Agrotis segetum* mixtures of *B.*

melolonthae-non-liquefasciens and blood from other normal caterpillars which have stood for some time (190).

Caterpillars have been employed in studying the possible effects of the nervous system upon the production of immunity. Caterpillars of *Galleria mellonella* which have had their cerebral ganglia and first two thoracic ganglia destroyed or which had been decapitated or deprived of head and first two thoracic segments all were immunized as easily as whole caterpillars (44, 128). Those in which the third thoracic ganglion was destroyed could not be immunized (128, 167). It should be noted, however, that this operation is a much more serious one for the caterpillars. Therefore, instead of interpreting this experiment as showing some peculiar importance of the third thoracic ganglion in the production of immunity, it seems possible that the loss of these ganglia has such a serious general effect on the caterpillar as to interfere with most of its physiological functions, including the production of immunity. When tight ligatures are placed around bee-moth caterpillars they continue to live 2 to 3 weeks, the posterior portion usually outliving the anterior portion. The two portions could be separately immunized, so that by proper selection of controls and by immunizing the two portions separately, results were obtained which seemed to indicate that the immunity was passed through the ligature by means of the nerve tracts (63, 64, 130, 131). It is easy to concede that some immune principle may have passed along the nerve tract, since to be alive nerve tracts would need to receive some blood supply. It seems, therefore, unnecessary to postulate a purely nervous transfer of the immunity.

Claims have been made that acquired immunity is hereditarily transferred from one generation of bee-moth caterpillars to another (126, 128). The experiment was done by immunizing a portion of each generation of caterpillars two or three times by injecting very small doses of cholera vibrios and then injecting all of them with minimal lethal doses of the same organisms and after 3 or 4 days counting the living and dead caterpillars, and calculating the percentage of the former. There was a rapid increase in the percentage of living caterpillars after two generations. This was interpreted as an inheritance of acquired immunity. It would seem more likely that the percentage of naturally immune individuals increased from one generation to the next by natural selection. The more susceptible individuals would die and fail to pass their higher degree of natural susceptibility on to the following generation. As mentioned in the section on natural immunity, the percentage of individual *Culex* mosquitoes susceptible to avian malaria

has been found to increase or decrease from generation to generation according to whether progenies were taken from susceptible or non-susceptible individuals (87).

Some puzzling examples of acquired immunity to chemicals, comparable to drug resistance in protozoa, are known in scale insects. Thus the San José scale (*Aspidiotus perniciosus*) and the citrus red scale (*Chrysomphalus aurantii*) are known to develop races resistant to lime-sulphur sprays and hydrocyanic acid fumigation respectively (115, 160). The mechanism of this newly acquired resistance is no better understood than it is in protozoa.

Cellular immunity. The discussion of the mechanisms by which invertebrates acquire and maintain immunity to infective organisms and the products of the latter will be divided into two portions, dealing separately with cellular and humoral reactions. Cellular immunity, or phagocytosis, results from the activity of the circulating cells of the blood and of the fixed cells which are phagocytic. The latter, as we shall see, may be dispersed, loosely arranged, or may comprise definite organs peculiarly adapted to the phagocytic function.

The literature on the blood cells of invertebrates and their origins is too voluminous to be reviewed in detail here. The earliest works were concerned chiefly with morphological studies of these cells and with conjectures as to their origin, function, and method of replacement (48, 66, 71, 72, 100, 111, 113, 141, 163, 164, 182). More recently the attention has been turned to attempts to classify the blood cells of the various groups, but the major interest has been in the cells of insects (47, 58, 74, 77, 82, 93, 102, 114, 136, 159, 181). In view of the great differences of opinion which exist upon the subject of the origin and classification of the blood elements of vertebrates, a comparatively homogeneous group, it is not surprising that there is not unanimity upon similar questions in relation to the invertebrates.

The blood cells of invertebrates may, in general, be likened to the lymphoid series in vertebrates. With certain possible exceptions, such as the hemamebocytes of terrestrial oligochaetes which secrete hemoglobin (112), we may say that the erythrocyte series is missing in the invertebrates. Blood cells are almost invariably found where there is any kind of lymph, hemolymph, or blood.

We lack a satisfactory comparative study of the blood cells of invertebrates. Many of the classes have been very little studied. In other classes such as the Crustacea (171) and Annelida (15) excellent studies have been made of a few forms, but comprehensive studies of the

various orders within the class do not exist. The insects, and particularly lepidoptera, have received the greatest attention. Since most of the studies on immunity have also been made upon lepidoptera, our attention will be centered upon the blood cells of that group. Cameron (16) has recently given us a critical review on this subject and, for convenience, we shall adopt the nomenclature used by him. The primitive cells with deeply staining, round nuclei and small amount of cytoplasm are the *lymphocytes*. They are actively ameboid and were referred to in the earlier literature as amebocytes and more recently they have been called proleucocytes (76) and macronucleocytes (144). A continuous series of transitional forms connects these cells with the *leucocytes*. The latter are larger than lymphocytes, have abundant, lightly stained cytoplasm, with occasional granules and vacuoles, and are strongly phagocytic. They have also been called large amebocytes, micronucleocytes, and phagocytes. The *spherule cells* are characterized by the presence of numerous, large acidophilic spherules which practically fill the cytoplasm and which are eventually liberated from the cell and which rapidly disappear in the plasma. The *oenocytes* (also called cerodecytes (79)) are huge cells associated with the fat-body with homogeneous cytoplasm and deeply-staining nucleus. First described by Wielowiejski (182), their function has since been a matter of controversy (68). Such abnormal elements as *giant cells* and *teratocytes* will be discussed under cellular reactions.

The blood cells (of insects, at least) have their origin in the undifferentiated mesoderm of the embryo (181). They are replaced either by division in the blood or by special *lymphogenous organs* (48). The lymphogenous organs, or lymph glands, are constituted of lymphoid tissue formed of young cells showing the characteristics of the primitive blood cells, grouped in nodules and held in place by connective tissue. They are found along the blood sinuses, but their number and disposition are quite variable (12). Distinct from these are the *nephro-phagocytes;* they are large, fixed cells with one or two nuclei, ovoid or elongated, and are either generally distributed or concentrated in masses. They have the dual functions of excretion and phagocytosis. The *phagocytic organs* are formed of a reticular network, the meshes of which are filled with phagocytes identical in appearance and function with the non-granular leucocytes of the blood. They are usually situated in the pericardial space in such a position that the blood must traverse them in reaching the heart (102). It is not possible at present to be very definite about the distribution of these organs in the various classes of

invertebrates, because they have often been confused one with another and also because some classes have not yet been carefully investigated. Lymphogenous organs have been described in annelids (probably phagocytic, 102, 104), orthoptera (47, 49, 55, 56, 102), scorpions (103), crustacea (7, 8, 11, 13) and cephalopod molluscs (102), although in almost every case there is controversy over the function of the structures described. We definitely know that they are missing in most of the insects (102) and in some crustacea (Phyllopods, 52). Phagocytic organs have been described in nematodes (98, 140), annelids (chaetopods, and hirudinea, 51), crustacea (5, 7–13), myriapods (31), insects (47, 55, 56, 102), diplopods (6), prosobranch (48) and opisthobranch molluscs (31), but again it is not clear in all of these cases that the organs are phagocytic in function.

The classic studies of Metchnikoff (137) on phagocytosis in *Daphnia* clearly demonstrated the importance of this process in the defense of the organism, and pointed the way to the comprehensive study of the phenomenon in higher animals. He showed that a definite relationship exists between the activity of the phagocytes of *Daphnia* against the spores of *Monospora* and the ability of the *Daphnia* to recover from the infection. In his more comprehensive study (138), other invertebrates such as starfish and insects were included. Other investigators took up similar studies on other organisms, for example, the infection of *Talitrus* (CRUSTACEA) with *Oïdium* (75) and of *Gryllus* (ORTHOPTERA) with a coelomic gregarine (46). It was noted, however, even in these early studies (46, 138), that phagocytosis was lacking in some cases, particularly in cases of insects attacked by nematodes, fungi, and parasitic larvae of hymenoptera and diptera.

With the recognition of the importance of phagocytosis in vertebrates the study of the phenomenon in invertebrates was practically abandoned for about 25 to 30 years. We have had, however, in the past 10 to 15 years a revival of interest in the latter field and a recognition of the value of including the invertebrates in studies on the general phenomenon of phagocytosis. Most of this later interest has centered in the insects. Before turning to a discussion of it, we should, however, mention the few studies made in other classes. Phagocytosis has been shown to be important in protecting the marine annelid, *Polymnia nebulosa,* against the coelomic coccidian, *Caryotropha mesnili,* whereas it was inactive against a coelomic infusorian (166). It is known to occur in molluscs, where it is thought to have a normal excretory function which may, under abnormal conditions, be greatly exaggerated (14, 62). In star-

fish it has been shown that foreign substances injected into the coelom are phagocytosed and carried through the walls of the branchial tubules and deposited outside the body (50). In these cases, however, it is not known to what extent phagocytosis protects the animal against and during infection. The efficacy of the phagocytic organs (buschelformigen organe) of *Ascaris megalocephala* (NEMATHELMINTHES) in removing bacteria injected into the body cavity is shown by the fact that these microörganisms begin to disappear 4 hours after injection and are subsequently found in large numbers within the phagocytic organs (124). In the Lumbricidae phagocytosis is effective in removing all foreign and waste materials except fat (15). Such groups as the arachnida, myriapoda and the more primitive forms from rotifers down have been practically neglected in respect to the importance of phagocytosis in their defense.

The bee-moth, *Galleria mellonella,* particularly in its caterpillar stage, has come to play in invertebrate immunology a rôle similar to that played by guinea pigs or rabbits in mammalian immunology. The fact that it normally feeds upon beeswax led to its use in studies upon digestion of the tubercle bacillus (2, 116, 117, 119, 121, 122, 128). Although it has proved to be admirably adapted to laboratory rearing and to experiments of this kind, there is evidently no peculiar relationship between its ability to digest wax and its ability to phagocytose tubercle bacilli (82, 84). The phagocytes of caterpillars which do not live upon wax (*Pieris, Aporia, Macrothylacia, Lithosia*) dispose of tubercle bacilli as readily as do those of *Galleria* (83).

The most important rôle in phagocytosis in lepidoptera is played by the leucocytes (16, 43, 73, 82, 123, 129), and this is likewise true for bee larvae (177). Next in importance are the lymphocytes. The spherule cells occasionally are phagocytic; the oenocytes are not. Following experimental infection the numbers of lymphocytes increase from about 40 to 45 per cent to 80 per cent or more in the first 12 hours. The percentage of leucocytes decreases from 50 or 60 per cent to nearly 10 per cent in the same period of time (16, 123, 172). There is an appreciable increase in spherule cells, which then lose their spherules and disintegrate. There is very little change in numbers of oenocytes. The increase in lymphocytes appears to be due to hematopoietic stimulation and its rapidity to the fact that the cells increase only by division of existing blood cells which are all in immediate contact with the stimulating substance. This increased hematopoiesis is often accompanied, as in vertebrates, by an abnormally large number of mitotic cells, a phe-

nomenon described as *karyokinetosis* by Paillot (145, 147, 150), who attributed peculiar importance to it in immunity. The chief difference in phagocytic response in normal and immunized caterpillars is the increased rate of phagocytosis in the latter (16, 128).

No definite case of the production of specific opsonins in invertebrates has come to my attention. Certain marine invertebrates have been shown to have some normal opsonic power which is lost when the leucocytes are washed (162). It was possible, moreover, to raise the opsonic index in *Sipunculus nudus* (GEPHYREA) from 0.27 in normal animals to 1.06 in vaccinated animals (31).

Phagocytosis, to be effective, must dispose of foreign substances either by intracellular digestion or by segregation or elimination of the undigestible particles. Segregation by nodule or cyst formation is common in insects. It has been seen in crickets against nematode and gregarine parasites (47), in a bettle, *Dytiscus marginalis*, against a distome parasite (80), in a meal-bug, *Icaurus*, against a sporozoön (106), and in caterpillar larvae (*Galleria*, *Pieris*, *Aporia*) against tubercle bacilli. The nodule formation against tubercle bacilli has been likened to tuberculous nodules in mammals by one writer (128), but others have claimed that the reaction is non-specific (16, 84, 96), since aseptic foreign bodies elicit the same response, and since these nodules in insects are simply formed of layers of leucocytes around the phagocytosed material (82) and that giant cells, true connective tissue, and caseation are absent (16). The claim that nodule formation is especially effective in disposing of living microörganisms (128) is also met with the opposite finding that the living organisms can be cultivated from the nodules during the remainder of the life of the insect (16, 82). Segregation of material in earthworms has been shown to be effected by transference of foreign substances to the terminal segments of the worm which are then cast off by autotomy (15).

The importance of the fat-body (155, 156, 158), the various types of phagocytic organs, and the pericardial cells in immunity has not been intensively investigated. However, the pericardial cells have been shown to have particular importance in disposing of foreign substances in caterpillars (1, 16).

Structures called *giant cells* have been found around parasitic fungi, inert foreign bodies, and tubercle bacilli in *Galleria* (3, 96, 128) and around leprosy bacilli in *Galleria* and *Carausius* (135), but have not been seen in *Pieris* and *Aporia* having tuberculous infections (82). The same term has frequently been applied to cells found in lepidoptera

parasitized by hymenoptera. These cells, which have been called *teratocytes* (79), are 90μ to 100μ in size with a large polylobed nucleus composed of fine chromatin grains. While the endogenous origin of these cells has been defended by some writers (79, 81, 152), others have very convincingly shown them to be surviving cells from the serosa of the parasite which have continued to grow in the body of the host (97, 153, 168).

In some invertebrates which have no circulation of blood there exist curious ciliated structures, called *urns* because of their shape, which swim about in the blood and differentially select foreign particles from their own normal blood cells and agglutinate the former into a mass on their trailing ends where phagocytosis is effected by the blood leucocytes (53). These are found in certain species of *Sipunculus* and *Phascolosoma* (GEPHYREA). There are also fixed urns in the former genus and in the *Synaptidae* (ECHINODERMATA). Functionally similar organs, although differing greatly in structure, are the *ciliophagocytic organs* of annelids (51). Although all of these organs actively remove foreign particles from the blood, extensive studies have only been made on the part they play in immunity in *Sipunculus nudus* (31).

At this point it is noted that invertebrates show very little reaction to carcinogenic substances. Some of them are, however, susceptible to infection with *Bact. tumefaciens* which releases important cellular reactions (175). Neoplasms are formed in *Nereis* (ANNELIDA) following inoculations of these bacteria, but only in those individuals already having granuloma-like processes resulting from oöcyte degeneration. This tissue enters suddenly into proliferation, invades, destroys and replaces neighboring tissue (173). The infrequence of neoplasms in invertebrates has been attributed by one writer to the fact that the cells of this group retain their embryonic character (61).

Humoral immunity. The spectacular part played by phagocytosis in the immune processes in invertebrates, together with early failures to demonstrate humoral antibodies, was responsible for the idea which prevailed for some time that invertebrates are incapable of forming antibodies. During the past twenty years a number of undisputed examples of the production of such antibodies have been clearly demonstrated, and these two decades have seen a heightened interest in this branch of immunology. Limits of space will prevent a detailed discussion of this literature. It has, therefore, seemed best to present a table of the more important literature in this field, arranged in such a manner as to allow the reader to delve into such aspects of it as happen

TABLE 1

The literature dealing with humoral immunity in invertebrate animals

PHYLUM† (4)	SPECIES	AGGLUTINATION	PRECIPITATION	BACTERIOLYSIS	CYTOLYSIS (HEMOLYSIS)	ANTITOXIC IMMUNITY	COMPLEMENT
Nematoda	*Ascaris*			N+,− (124)			N− (31)
Annelida	*Aphrodite*						N− (18)
	Sipunculus	A+ (27, 31)		A+ (28)	A+ (29)		
Arthropoda Crustacea	*Eupagurus prideauxii*	N+ (rabbit, sheep rbc, (17) *Bact. coli*, cholera vibrio) A+ (*Bact. coli*) (20)	N+ (horse, *Adamsia* poison, rabbit serum) (17, 23) A+ (rabbit serum)		N+ (rabbit, sheep) (17) A+ (rabbit) (20)	N+ (*Adamsia* toxin) (32, 33, 34, 35)	
	E. bernhardus	N+, A+ (23)	N+ (23)		A+ (29)		N− (31)
	Pagurus striatus	N− (17)	N− (17)		N− (17)		N− (18)
	Carcinus maenas						N− (18)*
	Palinurus vulgaris						N− (18)
	Maia squinado	N+, A+ (sheep, rabbit rbc) (23) A+ (*Sipunculus* blood) (30) N−, A+ (25)			N, A− (23) A+ (24) A+ (*Sipunculus blood*) (30)		N− (31)
Insecta	*Melanoplus femur-rubrum*	A+ (69)					
	Bombyx mori		A− (67)				N− (78)
	Galleria mellonella			A+ (119, 125, 148, 186,187) A− (65)		A− (127) A+ (42, 43)	

	Agrotis			A+ (146)			
	Pyrausta			A+ (129)			
	Oryctes					A− (139)	
	Vanessa, Io, Chelonia						N− (78)
	Sphinx, Decticus						
	Orphania, Ephippiger						
Arachnida	*Epeira*				N+ (109)		
	Limulus	N+ (143)					
Mollusca	*Helix pomatia*	N− (21) A+ (21, 62)	N− (21) A+ (21)		N− (21) A? (21) A− (21)	N+ (45)	N− (21)
	Eledone		A− (60)				N− (31)
	Sepia	N+, A− (37)	N−, A− (37)		A− (57)		N− (18)
Echinodermata	*Echinus acutus*				A− (57)		N− (18)
Chordata	*Phallusia mammillata*		A+ (31)				N− (18)
	Ascidia mentula			N− (22)			N− (18)

* Complement-fixation N or A (19).

† Phyla Coelenterata, Platyhelminthes, Nemertea, Rotifera, and Polyzoa are omitted because of lack of *references* to work done on them.

N = natural antibody; A = acquired; + or − = *presence or absence*; numerals in parentheses are bibliographic references.

See text for cautions as to interpretations.

to interest him most (table 1), and also as to display certain general characteristics of the work. Great caution should, however, be observed against taking the results, so tabulated, at their face values, for in almost every case, qualifications need to be made in regard to the positive or negative results recorded. In truth, the question may even be rightfully raised as to the advisability of using the same words, such as bacteriolysis, as are used in mammalian immunology, for processes which in invertebrates are not definitely known to be comparable. We shall now turn to a discussion of such general conclusions as can be drawn, without attempting to give individual attention to many of the investigations upon which these conclusions are based.

Complement, as we know it in vertebrates, has not yet been demonstrated in any invertebrate. There are, however, examples of complementary action, that is, serums of invertebrates hemolytic in themselves are capable of producing hemolysis in a sensitized system in dilutions incapable of producing it on non-sensitized red cells. Thus, the serum of *Eupagurus prideauxii*, which is naturally hemolytic for normal sheep cells in low dilutions, will hemolyze sheep cells sensitized by anti-sheep serum in much greater dilutions (31). Another example is to be found in the behavior of arachnolysin in certain spiders (EPEIRIDAE). The arachnolysin from the eggs of *Epeira diademata* is inactivated by heating to 62°C., but it is reactivated by very small amounts of unheated eggs from the same species or from a closely related species, *Meta segmentata*, which do not contain the arachnolysin (108–110). Cantacuzène has raised the interesting question as to whether one of the essential constituents of complement may be missing in invertebrates (31). It has been shown that the blood of *Helix* is lacking in serum albumins (54). Could its lack of complementary action be due to such a deficiency?

As table 1 shows, natural antibodies are abundantly represented in the invertebrates. The sera of many different forms bring about agglutination, precipitation, and lysis of widely different antigens. These are, of course, non-specific, as shown in the typical case of the natural hemolysin of *Eupagurus prideauxii*, which can be absorbed by typhoid bacilli. Natural agglutinins are abundant, lysins less frequent, and precipitins quite scarce. The majority of these have been found to be thermolabile. In addition, a substance has been demonstrated in the serum of *Maia* which impedes the action of rabbit complement on a rabbit-sheep-hemolytic system (24, 26). Moreover, if this substance is absorbed by non-sensitized erythrocytes, it retards considerably their hemolysis by the serum of *Maia* vaccinated against the red cells. The name of "opposing antibody" has been suggested for this substance.

A number of highly thermostable substances are also found to occur naturally in invertebrates. A Sclerostome of the horse contains a crystallizable organic substance capable of hemolyzing the erythrocytes of the horse. Its hemolytic property is not all lost by heating to 120°C. (179, 180). Apparently similar substances in other worms (principally cestodes) have been found to be highly bactericidal to a wide variety of bacteria (99, 157, 178). A powerful bactericidal principle has been demonstrated in the stomachs of many arthropods (59). This substance withstands drying and temperatures up to 120°C., is not destroyed by tryptic digestion, nor by precipitation with alcohol or acetone. While these thermostable substances cannot be termed antibodies, it is clear that they must be effective in protecting the animals against the organisms likely to invade them.

Successful production, by infection or vaccination, of precipitins, agglutinins, and lysins possessing a certain degree of specificity has also been effected in a number of cases (see table 1). In a great many cases artificial vaccination seems not only to strengthen the natural antibody already present, but to impress a certain degree of specificity upon it. The appearance of the phenomenon of bacteriolysis in insects which have been vaccinated is one of the clearest cases of artificially produced humoral immunity (125, 129, 146, 187). The striking difference, however, between these bacteriolysins and the bacteriolysins as we know them in vertebrate immunology lies in the fact that the former cannot be divided by heating into two portions (148). Not all bacterolytic power is lost even at 75°C. If we are to apply the term "bacteriolysin" to these substances, we shall need to broaden our concept of this phenomenon.

Experimental demonstration of antitoxic action in invertebrates has failed for the most part because of lack of susceptibility of invertebrate cells for known toxins (127, 139). Diphtheria toxin has, however, been found to be toxic for caterpillars of *Galleria mellonella*, and an immunity has been produced in the latter by the use of anatoxin (42, 43). An antitoxic immunity has been shown to exist in *Eupagurus prideauxii* against the substance in its coelenterate parasite, *Adamsia palliata*, which is highly toxic for many crustacea, echinoderms, molluscs and sipunculoidea (32 to 35). It is impossible at present to determine whether this is a case of natural or acquired immunity, since all crabs of this species are parasitized.

The reactions of agglutination or lysis which occur in the proximity of cells, even though the same reactions do not occur in the blood of animals, have been called "reactions of contact" (31, 38, 40, 151). They make excellent cases for speculation on the evolutionary origin

of humoral reactions in animals, but the number of facts we possess in regard to them is insufficient to be conclusive as to their nature.

Hypersensitivity. Very little attention has been paid to questions of hypersensitivity and anaphylaxis in invertebrates. A hypersensitivity in bee moth and other caterpillars develops after vaccination with cholera vibrios (133). Anaphylaxis has been experimentally produced in crayfish by the use of human serum (105) and in earthworms by rabbit serum (161).

CONCLUSIONS

In the invertebrates a natural immunity to the attacks of many infective organisms and their noxious products is the first line of defense. This natural immunity is poorly understood, but it is the result of complex processes, including phagocytosis and natural antibodies, many of which are probably inherent. Phagocytosis plays a spectacular part in immunity and, as in vertebrates, is effected both by free and fixed phagocytic cells. It is ineffective in some cases. There are examples of acquired immunity—both active and passive—in invertebrates, but the specificity of such immunity is open to some question. Antibodies may be produced following vaccination or infection, but these result most successfully when natural antibodies already exist. These antibodies differ widely from the corresponding ones in vertebrates, as exemplified by the bacteriolysins which are highly thermostable. No complement has been found, but complementary actions occur. It is too early yet to attempt to evaluate the relative parts played by the various immune mechanisms in protecting the invertebrates.

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THE FUNCTIONAL SIGNIFICANCE OF SPECIFIC AGGLUTININS AND PRECIPITINS

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It has become increasingly evident within recent years that antibodies are not the mysterious agents they were once supposed to be, but are, in fact, serum globulins which have been specifically modified in response to antigenic stimulation (1, 2, 3). Why they appear is not known. Zinsser has suggested that they probably arise during immunization because of the presence in the tissues of foreign, non-diffusible colloids which, in order to be excreted or utilized, must be broken down into diffusible forms (4). If this is true the immune reactions may be considered as the outgrowth of an evolutionary biological digestive mechanism developed for maintaining the specificity of the body's proteins (5); in the processes of immunity "the many different sorts of proteins are disintegrated into their building stones, the amino acids, and so the individuality of the food proteins is lost before their absorption" (6).

Although many descriptive names have been given to antibodies (agglutinins, precipitins, opsonins, lysins, antitoxins, sensitizins, etc.) it is probable that they all are basically identical in nature (7, 8, 9). This has become more apparent with the use of purer antigens in immunization and the development of more accurate quantitative methods for the study of antibodies and of antibody-action. The prevailing view is that specific antibody globulin combines with antigen, whether particulate, or molecularly dispersed, and forms a deposit of antibody-protein on the antigenic surface. This deposit, by altering the surface properties of the antigenic elements, increases their cohesiveness and viscosity so that mutual contacts lead to adherence (10, 11, 12). The earliest effect, therefore, is aggregative or coagulative, whether the antigenic units are in red blood cells, trypanosomes, colloidal micelles, toxins or elementary bodies, and may be regarded as the initial step in the conversion of foreign colloids into simpler, more assimilable forms. This coagulative action resembles somewhat that of rennin in the early

stages of gastric digestion but differs in its extremely sharp chemical specificity.

Recent evidence strengthens the long-held assumption that agglutinins and precipitins are identical. Jones (13), and Hulshoff Pol (14), for example, have shown that when proteins are adsorbed to particulate surfaces (collodion, collophonium, kaolin, etc.), the sensitized particles agglutinate when suspended in their specific antiserums. Delves has demonstrated, furthermore, that if precipitins are specifically absorbed from an antiserum, collodion particles sensitized with the specific protein will not agglutinate when suspended in the antiserum (15). Heidelberger and Kabat (16), by gravimetric methods, and Francis (17) by serologic ones, have recently proved the identity of type specific precipitins and agglutinins in antipneumococcic horse serum. Of even more interest is the close relationship between agglutinins and precipitins, and other types of antibodies. It has been shown that the addition of a specific antiserum to a mixture of washed leukocytes and collodion particles sensitized with protein promotes phagocytosis (18, 19), but that removal, by specific absorption, of specific precipitins against a purified protein removes the ability of the serum to promote phagocytosis of collodion particles sensitized by this protein (15). In pneumococcic infections of mice, serum protection parallels its maximally precipitable antibody globulin (20, 21, 22). Injection into normal guinea pigs of the washed precipitate from the interaction of a protein and its antiserum confers passive sensitization to anaphylactic shock (23). Finally, bacterial toxins (24) and filterable viruses (25) have been adsorbed to the surfaces of collodion particles; when these particles were mixed with their respective antisera, flocculation or neutralization, or both, occurred. All these secondary manifestations of antibody action, therefore, may be looked upon as subsidiary to the primary attachment of antibody globulin to the antigenic particle with the resulting changes in its surface properties. The aggregative process, once initiated, is followed by the progressive phases of dispersion (lysis, intracellular digestion) which are later stages of hydrolysis or digestion occurring outside of, or within, cells.

The complicated mechanism of agglutination has been extensively studied in the test tube. The present view is that aggregation of particles in suspension results from their cohesion after chance contacts (26, 27, 28). The number of contacts and the degree of cohesion are influenced by the mass, size and number of particles per unit volume, Brownian motion, viscosity and temperature of the dispersion fluid,

electrolyte and lipin content and other factors. In the presence of specific antibody the deposition of antibody globulin, by increasing the cohesiveness of the particulate surfaces, tends to overcome the so-called surface potential of the similarly charged particles, and leads to aggregation. Although there is still doubt as to the relative importance of physical and chemical influences (29), there is fairly general agreement about the main features of the process. The precipitation reaction is assumed to develop similarly, differing only with respect to the size of the antigenic elements which combine with antibody (30).

Are similar forces interactive in the body? Early workers attributed little if any importance to agglutinins and precipitins in acquired immunity and some even doubted their union with antigen *in vivo*. While Metchnikoff (31) believed that "the part played by agglutination in immunity can only be very inconsiderable" and that it is "merely accidental and subordinate," his early statement that "it is probable that in certain special cases the immobilization of very motile bacteria and their agglutination into clumps may facilitate the reaction of the animal organism, especially of phagocytosis" is of interest because it foreshadows the present-day view that agglutination, on the one hand, or opsonization and phagocytosis, on the other, depend largely upon chance contacts, either between the bacteria themselves, or between bacteria and phagocytes. Weil, in 1917, said (32), "All in all, it is apparent that agglutination plays less of a rôle in the processes of disease than might be assumed *a priori*, judging from the effectiveness of the test-tube reaction"; and of precipitation he remarked, "there are no observations which permit of a conclusion as to whether it actually takes place in life. There are some authorities who seriously doubt this occurrence, and yet it seems difficult to justify this doubt. Soluble proteins, which are the material of precipitation, cannot, of course, undergo adaptive alteration like the bacteria and become non-precipitable. It is quite likely, however, that microscopic precipitates may be filtered out by the phagocytes as rapidly as formed, and that actual gross flocculation does not, therefore, occur. This would explain the fact that it has never been possible to demonstrate the phenomenon in the circulating blood." Bailey, a decade later, in his review of the functional rôle of agglutinins (33) said, "Agglutination has been considered by most immunologists to occupy a subordinate place; for, however valuable the phenomenon may be *in vitro* in identifying bacteria or in discovering specific forms of infection, it is held that the process plays no essential part in the protection of animals." It is true, how-

ever, that a few early workers were impressed by the possible importance of agglutination in protection against infectious disease. Thus, Sawtschenko and Melkich (34) injected spirochaetes of relapsing fever into the peritoneal cavities of immunized guinea pigs and observed that within from ten to fifty minutes the spirochaetes became immobile; "les spirilles se réunissent en petits amas, s'agglutinent." They also saw occasional agglutinated masses of spirochaetes in blood taken from patients with relapsing fever, but pointed out that the rapid movement of the blood would naturally prevent marked agglutination. Wright and Lamb (35) noted, in guinea pigs infected with plague bacilli, that the organisms were uniformly dispersed in the blood throughout the body, whereas if immune serum was injected, the bacilli were found only in discrete colonies in the spleen and other organs. It seems strange, in retrospect, that at a time when there was so little doubt about the importance of lysis, antitoxic action, opsonization and anaphylactic sensitization, so many should have questioned the occurrence of agglutination and precipitation *in vivo.* But comparatively little more attention was given to the subject until Bull, in 1914, suggested that agglutination was a more important mechanism of defense than had been generally supposed (36). He injected living typhoid bacilli into rabbits and found that the microörganisms were not killed in the blood stream but were rapidly removed from the circulation. They became concentrated particularly in the liver and spleen, occurring in clumps in the capillaries and sinusoids, and were engulfed by polymorphonuclear leukocytes which also accumulated there. When he injected specific immune serum into rabbits during the bacteriemic phase of pneumococcic infection, the pneumococci disappeared rapidly from the blood stream and accumulated in agglutinated masses in the liver and spleen where they, also, were quickly engulfed by the leucocytes. The degree of agglutination appeared to vary inversely with the infectiousness of the bacteria, and less infectious organisms might even agglutinate spontaneously; in any case the liver and spleen acted as the principal filtering organs in which the phagocytes destroyed the microörganisms. These observations merit renewed attention in view of Knisely's recent studies (37) on the circulation of the spleen. He has shown that the splenic sinuses in the mouse may become distended in the "filtration-filling" phase and may remain so in the "storage" phase for considerable periods of time. It is reasonable to suppose that in such areas of stasis in an immune animal, leukocytes and bacteria simultaneously entrapped might have unusually favorable opportunities

for contact, thus favoring agglutination, phagocytosis, or both. Zinsser, a few years later, corroborated the observations of Bull (9) by injecting type 1 antipneumococcal globulin solution intracardially into rabbits in the septicemic phase of pneumococcic infection and then examining smears of blood withdrawn from the heart a few minutes later. In the smears he observed clumps of pneumococci which, he believed, indicated agglutination in the blood. Rheingold injected suspensions of B. prodigiosus intravenously into dogs and came to similar conclusions (38). He found aggregates of bacilli in the capillaries and sinusoids of the liver, "indicating that agglutination had occurred in the blood stream." He attributed the removal of the bacteria from the blood to this agglutination in the capillaries, chiefly of the splanchnic area, followed by phagocytosis and digestion of the microörganisms. Further evidence supporting the conclusions of Bull is furnished by the experiments of Manwaring and his associates (39, 40). They found that, when organs of normal and of immune animals were perfused with Ringer's solution containing living pneumococci, the livers of the normal animals showed but little tendency to remove the pneumococci whereas those in immune animals quickly removed the microörganisms. Smears and histological preparations showed numerous pneumococci adherent to the capillary endothelium. Taliaferro and Cannon (41) also observed, in their study of the problem of superinfection in malaria in monkeys, that the malarial parasites accumulated particularly in the spleen and became concentrated in the cords of Bilroth, as if they had been made more adhesive or had been agglutinated by the action of the immune bodies, after which they were aggressively phagocytosed by macrophages. From the foregoing it is obvious that antibody can combine with antigenic materials in the blood and influence their surface properties so that they may adhere to one another, to phagocytes or to the tissues. It is interesting to recall that the name, agglutinin, arose because of Gruber's belief that this antibody affected the bacterial membrane, causing it to become "klebrig" or glutinous (42).

The failure of many of the earlier workers to observe these changes in bacterial surfaces in the body is not surprising in view of the difficulties inherent in the study of agglutination in the blood stream. Probably not much would be known about the principles influencing the mechanism of agglutination if the study had been restricted in the past to the observation of the effects of antibodies upon a suspension of bacteria in a circulating pump; we know today that bacteria cannot agglutinate unless they can come into contact with one another, and

opportunities for mutual contact in the circulating blood are certainly not favorable. And even if such contact should occur in sinusoidal areas of blood stasis, phagocytosis might quickly remove all evidences of beginning agglutination. In order to avoid these difficulties, more recent workers have endeavored to observe the bacterial changes, by histological and cultural methods, after injection of microörganisms into the subcutaneous and other tissues of normal and of immunized animals. Morphologic observations, at varying intervals, of the ensuing inflammation afford an excellent opportunity to follow the sequence of events. By such methods bacterial localization in immune animals and the changes in the bacteria themselves have been studied.

Tsuda (43) injected virulent streptococci and pneumococci into the skin of normal and of immunized mice and noted that, in the non-immune animals, the inflammation was a spreading type, whereas in immune animals the inflammatory reaction was more circumscribed and the microörganisms remained localized near the site of introduction. Although he was more interested in the inflammatory reactions than in the changes in the bacteria themselves, he made the important observation, that, "wenn die Immunität stark genug ist zeigen die injizierten Kokken an der Injektionsstelle Agglutinationserscheinungen in Form von flockiger, zusammengeballter Masse." Bass (44) introduced hemolytic streptococci into the tibial bone marrow of normal and of immunized rabbits; in the former only moderate phagocytosis by histiocytes and polymorphonuclear leukocytes occurred and the microörganisms caused death by septicemia. In the immune animals, however, agglutinated cocci were seen, followed by abundant phagocytosis and no serious consequences to the host.

Cannon and Pacheco (45, 46) injected suspensions of living virulent staphylococci intradermally into normal and locally vaccinated guinea pigs. Histologic examination revealed early and marked agglutination of the staphylococci in the immune animals, followed by active phagocytosis by leukocytes and macrophages. In the normal animals the microorganisms spread diffusely through the subcutaneous tissues, despite inflammation and phagocytosis. They concluded, therefore, reasoning from the experiments of Opie on the nature of the Arthus reaction, that the primary bacterial localization was effected by an immediate antigen-antibody union and was maintained by phagocytosis and a more abundant infiltration of cells of inflammation. They assumed that the antibody-antigen combination also increased the efficiency of phagocytosis through its opsonizing action and that the

summation of all of these influences promoted not only the immediate but also the continued localization of the infectious agents.

Rich and his associates (47, 48), in their studies on the rôle of immune bodies in pneumococcic infection, demonstrated clearly the prompt adherence of the microörganisms to the immune tissues. When living pneumococci were injected into the skin of rabbits passively immunized with type-specific homologous antiserum, the microörganisms remained sharply localized and grew into skein-like colonies in the subcutaneous tissues. The degree of bacterial localization varied inversely with the amount of antiserum administered, so that with lesser amounts of serum the lesions were more diffuse, edematous and hemorrhagic. The localization occurred before there were marked evidences of inflammation, and even in animals deprived of circulating leukocytes by the previous administration of benzol. Here, however, the localization was but temporary, and eventually the pneumococci entered the blood stream and caused death by septicemia. Rich concluded from these and other experiments that the prompt bacterial localization in immune tissues is not caused by inflammation, but is due to the primary interaction between bacteria and immune bodies. This leads to an increased adhesiveness of bacterial surfaces which causes the bacteria to adhere to the tissues and to one another, thus hindering their free "drift" through the tissue spaces.

Catron (49) injected type 1 pneumococci into the subcutaneous tissues of normal and of actively or passively immunized mice. Histologic examinations revealed sharp localization of the pneumococci in the latter animals; "agglutination, occurring within 5 minutes of infection, assured localization of the bacteria for a period during which they continued to proliferate," whereas in the normal animals although there was widespread inflammation, the bacteria spread diffusely through the subcutaneous tissues, and were not phagocytosed. Catron concluded that agglutination in the subcutaneous tissues was an important early localizing phenomenon but that phagocytosis was the means whereby the pneumococci were destroyed; "thus localization and destruction of the bacteria in immunized mice were dependent both on bacterial changes caused by specific antibody and on phagocytic activities of host cells."

Cannon and Hartley (50) suspended living virulent pneumococci in egg white solution and injected them subcutaneously into rabbits both actively immunized against this strain of pneumococci and sensitized against egg white. A histological study of the ensuing lesions

showed that the microörganisms multiplied for a time in the developing area of anaphylactic inflammation and showed unmistakable evidences of capsular swelling as if undergoing a Neufeld "quellung" reaction in living tissues. Small clumps of pneumococci were seen as well as larger, compact colonies, indicating the influence of antibody on their surfaces and the resulting tendency for the bacteria to remain localized. Such animals apparently suffered no ill effects from the pneumococcic infection, whereas normal controls, and controls sensitized against egg white but not immunized against pneumococci invariably died from septicemia within 48 hours.

Gins, Kroemer and Link (51) have recently described the early changes in the subcutaneous tissues of normal and immune guinea pigs infected with Welch bacilli, and a pathogenic strain of B. coli. The Welch bacilli, in normal animals, grew diffusely through the subcutaneous tissues whereas, in immune animals, they were seen "vorwiegend in kleinen Häufchen, wie agglutiniert, so weit sie nicht phagozytiert sind." With the colon bacilli, in immune animals, the organisms were seen within one and a half hours in "einzelnen Stellen in lockeren Herden frei im Gewebe, meistens sind sie in dichten Häufchen zusammengedrängt so dass der Eindruck einer Agglutination im Gewebe entsteht." In both types of infection, besides the agglutinative phenomena, phagocytosis, swelling and disintegration of the bacteria, sharp localization of the lesion and speedier resolution of the localized areas of infection were observed in the immune animals. Hammerschmidt (52) has also shown, by histologic methods, that, when virulent organisms of mouse septicemia were mixed with agar and injected subcutaneously into normal, and passively immunized mice, the development of the bacteria in the agar foci was strikingly different. In the normal animals the bacteria grew well, wandered out of the agar and into the surrounding tissues and then invaded the body as a whole. In the immune animals they grew into sharply circumscribed colonies and bacteria were not seen in agar between individual colonies. Hammerschmidt was apparently not aware of the work of Rich and gave no consideration to the probability that the changed surface properties of the organisms in the immune tissues may have increased their tendency to adhere to one another and thus to grow into compact colonies. Instead, he concluded that this localization was due to the inhibitive action of antiaggressins; his illustration, however, shows the sharp localization of the bacteria in the agar focus, the agar being surrounded by a zone of leukocytes.

These experiments indicate that agglutinins are important factors in the early localization of bacteria in tissues and suggest certain ways in which they act. Inasmuch as, under natural conditions, the number of microörganisms entering a tissue is limited, the first effect is probably that emphasized by Rich, namely, adherence to the tissues themselves. Even though the bacteria continue to grow, this primary localization allows time for the inflammatory reaction to develop. As the bacteria increase in numbers and drift from the primary area of localization, particularly if edema is developing, contacts with one another presumably favor agglutination. This agglomerative tendency may not only restrict the movement of the larger clumps through the tissue spaces, but may also, through its opsonizing action, enhance phagocytosis. For example, the effectiveness of phagocytosis must depend necessarily upon the number of contacts between phagocytes and bacteria. If a phagocyte, at one contact, can engulf a clump of a dozen or more agglutinated bacteria, whereas, in the absence of clumping, twelve separate contacts would be required to accomplish the same effect, the advantages of an early agglomerating action are apparent. And even though phagocytes should engulf such large clumps as to make intracellular digestion difficult, the microörganisms, nevertheless, would be in one mass within a cell, rather than left free to enter the lymph and blood streams in large numbers.

The effectiveness of this agglomerating antibody-action is doubtless dependent upon the availability of specific antibodies in the blood and tissues and the kinds and numbers of bacteria invading. It is possible that if large numbers of bacteria with great growth energy and an ability to form large amounts of capsular combining material enter the tissues, agglutination may be less effective and the bacteria can grow and spread freely into the blood and lymph. The so-called aggressins may act in this way to nullify the combining potentialities of immune bodies. Variations in invasiveness, therefore, could be related directly to the ability of the microörganisms to produce and liberate combining materials. Under more favorable circumstances, as for example, with a large amount of antibody available in the tissues, combination of antibody with the bacterial surfaces should favor early immobilization, opsonization, phagocytosis and sharp localization of the lesion. Certainly one of the most dramatic experiments of acquired immunity is the demonstration that merely the presence of an adequate amount of type-specific antibody in the tissues can cause the normally extremely susceptible rabbit to react to the intradermal injection of

highly virulent pneumococci as if they were saprophytes, whereas in the non-immune animal the pneumococci will spread freely from the point of entrance and quickly lead to septicemia and death.

This localizing potentiality of immune tissues, and the accelerated inflammatory reaction usually accompanying it have long been known. Jenner observed the latter a century before the word allergy had come into use (53); after the demonstration of the Koch phenomenon and the development of the tuberculin test, many workers in the field of tuberculosis studied the problem of reinfection. From their experiments conflicting views have arisen concerning the significance of the local lesion, i.e., whether it results from an intensified inflammatory reaction which "walls off" the infecting agents by a mechanical barrier of leukocytes and fibrin; whether it is due mainly to effective phagocytosis, or whether it is the result of a primary union of bacterial antigens and antibodies, reinforced by inflammation and phagocytosis (54, 55, 56, 57, 58). The various aspects of the subject have been extensively reviewed within recent years and will not be discussed here (59, 60, 61, 62, 63). Opie has stated the problem in these words, "antibodies, such as precipitins or agglutinins, may have a part in the local fixation of foreign protein or bacteria" (64), but "in this process of local fixation it is not possible to estimate the relative importance of antibodies, of phagocytes and of the inflammatory reaction itself" (65). It should be pointed out, however, that the conflicting views concern only the question whether bacterial localization results *primarily* from the interaction of bacteria and antibodies or whether it is accomplished primarily by the inflammatory reaction itself through the deposition of a network of fibrin and the thrombosis of lymphatic vessels. The latter viewpoint has been discussed recently by Menkin and will not be considered further here (66). At any rate, the numerous studies of this problem have aided greatly in clarifying several phases of the subject and have revealed important features of the mechanism responsible for the early localization.

As with the agglutinins and antigen, the problem of the functional significance of precipitins was similarly a perplexing one for many years, and, because earlier workers so frequently observed the coexistence of antigen and precipitin in the blood, some even doubted that they can combine in the body to form a precipitate. Later experiments, however, proved that the usually slow disappearance time of a protein from the blood is markedly accelerated when precipitins start to appear and that if precipitins are already present, the protein may disappear

very quickly (67, 68, 69). As Culbertson has said, (70), "immunized animals which possessed no circulating precipitins at the time of the test injections of antigen required more time to dispose of a given amount of horse serum than did animals which possessed circulating precipitins." Later, presumably as a result of the anamnestic reaction, removal of injected antigen was more rapid, even in animals previously immunized but no longer containing circulating precipitins, than in animals injected with horse serum for the first time.

The uncertainties of the earlier workers were due largely to the use of complex proteins; horse serum and egg white contain multiple antigens (71, 72, 73, 87), and because of the multiplicity of antibodies engendered, quantitative identification is difficult. Results became more clear-cut when purer antigens were used. Opie (77) injected crystalline egg albumin into rabbits immunized against it and at no time observed the coexistence of this antigen and its precipitin in the circulating blood; instead, there was a marked diminution in or a temporarily complete disappearance of precipitins from the blood.

Culbertson (70) also injected crystalline egg albumin into the blood stream of immunized rabbits and found that the rapid removal of the albumin was accompanied by a marked decrease in the precipitin content of the blood. Proof that this was due to an antigen-antibody reaction was demonstrated by the fact that the intravenous injection of nonspecific materials such as normal horse serum, cow's milk, peptone, bacterial vaccines, etc., caused no significant changes in the precipitin content for crystalline egg albumin. Although he recognized the potential combining ability of fixed-tissue precipitin, his calculations convinced him that the circulating precipitins played the predominant rôle in the elimination of crystalline egg albumin, and that the fixed-tissue precipitin functions "only when some of the antigen escapes union with the circulating precipitin and reaches the fixed tissues." Hektoen and Welker (74) showed clearly that the negative phase, i.e., the reduction or complete disappearance of antibody from the blood after introduction of the specific antigen, is sharply specific and that "in the rabbit immunized against many antigens the injection of one of the antigens as the rule resulted in the disappearance from the blood of the precipitin for that antigen only." They quote Oguchi and Hamano as having found, also, that when a serum fraction was injected into an animal containing multiple precipitins, only those to the one fraction injected, disappeared.

Although no one has demonstrated that a precipitate actually forms

in the blood and that it is removed by phagocytosis, as suggested by Weil, there is indirect evidence that this may occur. Cromwell and Centeno (75) observed that vacuolated mononuclear cells appeared in the peripheral blood of rabbits immunized against crystalline egg albumin after its intravenous injection whereas this did not happen with normal rabbits. Inasmuch as they found a similar vacuolation in leukocytes added to a mixture of protein and its antiserum, but no such effect when the leukocytes were added to a protein mixed with normal serum, or to a mixture of antiserum and protein after removal of the precipitate, they concluded that the vacuoles were associated with phagocytosis and digestion of the specific precipitate. Opie also showed (76) that when washed precipitate from the union of antigen and antibody was injected under the skin of a normal rabbit, leukocytes accumulated and eventually removed it.

Evidence for the occurrence of precipitation in the tissues has been furnished largely by the experiments of Opie (77, 78, 79). When he injected crystalline egg albumin into the skin of a normal rabbit he observed that it quickly diffused from the site of injection, but that with each succeeding injection, at intervals of a few days, more was retained at the injection site. With the development of the phenomenon of Arthus, most of the injected antigen was retained locally. Furthermore, if antigen was injected into the blood of a normal rabbit, and, some hours later, specific antibody was injected into the skin, local inflammation occurred. He concluded, therefore, that the local retention of antigen is due to the local union of precipitin and antigen, aided by the inflammatory reaction and that "the behavior of precipitins in the living body is similar to that in the test tube and indicates that they precipitate foreign protein introduced into the immune animal." As a consequence of this local fixation of antigen, local destruction of the precipitate occurs. Opie concluded that the injury of tissue, the edema, hyperemia, hemorrhage, thrombosis and necrosis, could all be explained by the union of antigen and antibody outside of and within cells. In this process, however, generalized anaphylaxis, from absorption of antigen, was largely prevented, the local tissues, instead, bearing the brunt of the reaction. Dienes (89) also found a definite relationship between the presence of precipitins against egg globulin, ovomucoid, and crystalline egg albumin and the intensity of the delayed hypersensitive cutaneous reactions to these protein fractions, indicating a sharply specific antigen-antibody union of the individual antigens.

These experiments point strongly to the importance of precipitins

in restricting the spread of a protein, after it has become toxic to hypersensitive tissues, to more vital organs, and indicate that the efficiency of the localizing process varies directly with the presence and concentration of specific precipitins in the circulating blood. Opie showed that the Arthus reaction has a relationship to precipitins, also, in that it can be readily produced only in animals which can liberate precipitins freely (rabbit, goat) and can be demonstrated poorly or not at all, in animals which are poor precipitin-formers (rat, dog). He showed, furthermore, that in rabbits in which the phenomenon of Arthus is demonstrable, "desensitization," by the injection of a large amount of antigen, leads to a diminution in precipitative potency of the serum and a loss of skin reactivity. Opie concluded, therefore, that there is a close although not exact parallelism between cutaneous reactivity and precipitative power of the blood serum. Culbertson, in repeating Opie's experiments but using a better quantitative method for the determination of precipitins, concluded that "all of the evidence obtained indicates that tissue hypersensitiveness in an actively sensitized animal is dependent upon the formation of antibody by the animal and is reflected by the presence of precipitin in the circulation. The tissue sensitizing substance in an antiserum appears to be inseparable from and, probably, identical with the precipitin."

The above facts would be completely convincing were it not that several other workers have failed to observe any relationship between precipitative strength of the serum and intensity of cutaneous reactivity to the same antigen (81, 82, 83, 84, 85). Kahn, in particular, stated with respect to skin reactions in rabbits sensitized to a protein that "with time, the serum precipitins disappear from the circulation, while the capacity of the animals to give a skin sensitivity response remains" and that the parallelism between the two was of comparatively short duration. He concluded that "tissue hypersensitiveness is a more permanent biologic response than precipitin production and that the two phenomena are independent of one another."

These opposing views suggest that the conflicting findings may be due to differences in the experimental methods used. Many of the workers used complex antigens, such as serums and bacterial suspensions, despite the long-time insistence of Wells that immunologic principles can best be discovered and understood by the use of simpler and purer antigens. It is doubtful whether fundamental principles of chemistry could have been developed if chemists had continued to disregard the state of impurity of their reagents. It is true, of course,

that we do not deal with pure antigens in nature and that many important immunologic facts have been secured by the use of complex materials, but it is also true that the essence of the scientific method is to have as many elements as possible of a problem controlled.

Perhaps the most important source of error in evaluating the functional significance of precipitins is the method of determining the precipitative potency of an antiserum. The method almost universally used is known as the antigen-dilution method, in which serial dilutions of antigen are mixed with constant quantities of antiserum. This is the reverse of the usual serological method for the determination of antibody-strength of a serum, where serial dilutions of the serum are made and the highest dilution exerting a specific effect is characterized as the antibody-titer.

Culbertson has pointed out that the antigen-dilution method is absolutely inaccurate as a quantitative measure of precipitative strength of a serum and is, in fact, not a method for the titration of antibody at all, but only of antigen. Opie recognized this fact when he said that "the precipitin titer is not an accurate measure of the precipitin content of a serum." Only since the development of more accurate quantitative methods by Heidelberger and Kendall (86), Culbertson (87) and others, has it been possible to determine the actual precipitin content of an antiserum, expressed as milligrams of precipitated protein. By the use of this method Culbertson has confirmed the conclusions of Opie with respect to the rôle of precipitins in the development of the phenomenon of Arthus. Cannon and Marshall (88), using a serological method which determines the precipitative potency of an antiserum by dilution of the serum, have obtained results in complete agreement with those of Opie and of Culbertson.

Further evidence of the functional importance of precipitins is furnished by the experiments of Blacklock, Gordon and Fine (89), employing fly larvae, Cordylobia anthropophaga in immune and non-immune guinea pigs. They found that in immune animals about 80 per cent of the first instar larvae which penetrated the skin died within 40 hours and that many of these dead larvae "had their posterior end covered by an adherent cap consisting of semi-disintegrated cells lying in a homogeneous matrix." At an earlier stage these larvae were surrounded by a loosely adherent whitish coagulum and their guts were distended with a mass of fine granules. Such larvae were dead or feebly moving, had not increased in size and were sometimes shrunken. When larvae from non-immune guinea pigs were placed in immune serum "a pre-

cipitate quickly formed round them and this precipitate gradually increased in density." These phenomena were not observed in larvae in the skin of non-immune guinea pigs nor when larvae were placed in normal serum. They found a correlation between the presence of precipitins in the hemocoele fluid and the excreta of the larvae in the serums of immune guinea pigs. They concluded, therefore, that "the precipitate which is always formed in the gut of, and round the larvae, in immune animals is due to the interaction of the animal's serum with the excreta of the larva" and that the "apparent blocking of the gut and the enveloping principle around the cuticle must hamper the normal development of the larva and may be the direct cause of the death of the parasite on the skin of the immune host."

More recently the important studies of Taliaferro and Sarles (90, 91) on the immunity of white rats to infection with Nippostrongylus muris have given clear-cut demonstrations of an important rôle of precipitins in acquired resistance. When the larvae of this parasite were placed on the freshly-shaven skin of normal rats, penetration and migration occurred within 20 hours, the larvae feeding, developing and passing to the blood stream, lungs, and into the trachea where they were swallowed and thus reached the upper part of the small intestine. In reinfection of immune rats, on the other hand, the larvae, after penetrating the skin, tended to remain localized as somewhat stunted, coiled and immobilized forms and "precipitates formed in and around them." During this stage a more intense inflammation developed than in the normal animals, and nodules formed around the larvae. Similar precipitates formed in and around the larvae which reached the lungs and intestines. Taliaferro and Sarles concluded, therefore, that "antibodies (precipitins and possibly other humoral factors) immobilize, form precipitates in and around, stunt and sometimes kill the worms. They also localize the irritating excretions and secretions of the worm and bring about more intense inflammatory responses."

The identity of precipitins and sensitizins was early suggested by the experiments of Doerr and Russ (92) in which the parallelism between the precipitin-strength of a serum and its sensitizing potentiality were shown. They reported that as rabbits were immunized against a foreign protein the precipitative potency of the serum varied directly with its anaphylactic sensitizing properties; that when a serum varied markedly in precipitative reactivity to heterologous antigens, anaphylactic sensitivity to these antigens varied in the same direction; that white mice cannot be sensitized, either actively or passively, to

anaphylactic shock, and neither can they form precipitins nor anchor precipitin to their cells. Lake, Osborne and Wells noted, in 1914, that they could transfer anaphylactic sensitivity passively as soon as precipitins had appeared in the serum of the donor, and suggested the identity of precipitins and sensitizins. Weil, as stated above, showed that the injection of the washed precipitate conferred passive sensitization; knowing that the bulk of the precipitate consisted of precipitin, he concluded that precipitins and sensitizins are therefore identical.

These facts have a direct bearing on the possible relationship of precipitins and sensitizins to those tissue reactions, the so-called allergic or hypersensitive states, in which the adverse effects are definitely pathological. The evidence presented thus far has pointed more to the beneficent features of the aggregative reactions; attention will now be directed to their harmful effects.

The accelerated removal of a foreign protein from the blood stream might be regarded merely as an interesting biological phenomenon were it not for the fact that therapeutic serums are so widely used in the treatment of infectious disease. Inasmuch as most of these are prepared by immunization of horses, their injection into individuals hypersensitive to horse serum, either naturally acquired, or through previous injection with horse serum, may lead to their rapid removal from the blood before they have been able to exert any marked therapeutic effect. That this has happened is shown by those instances in which persons who have been given a prophylactic injection of diphtheric antitoxin, and, later, have contracted diphtheria, have not responded favorably after reinjection of a therapeutic amount of diphtheric antitoxin. The explanation is furnished by the following experiments: Roemer and Viereck (93) observed that, in guinea pigs sensitized against horse serum, antitoxin injected into the blood disappeared more quickly than it did in normal animals; Lewis (94) found that diphtheric antitoxin, if injected into rabbits which had been previously injected with horse serum, was considerably less effective in neutralizing diphtheric toxin subsequently injected than in normal rabbits. Glenny and Hopkins (69), in their extensive study of this problem, came to similar conclusions, and because of the fact that the antitoxin disappeared from the blood of normal rabbits within from 7 to 8 days whereas, in rabbits hypersensitive to horse serum, it disappeared within from 3 to 4 days, coincident with the appearance of precipitins, they believed that the union of precipitin and antigen accounted for the more rapid removal of the antitoxin.

The absorption of antitoxin may be seriously interfered with, also, if the antiserum is injected subcutaneously or intramuscularly into hypersensitive tissues. Hartock and Schürmann (95) showed that antitoxin injected subcutaneously into guinea pigs sensitive to horse serum exerted a considerably less protective effect than it did in normal guinea pigs. They made the interesting observation, also, that if guinea pigs sensitive to horse serum were injected subcutaneously with small amounts of horse serum to induce a state of anti-anaphylaxis, antitoxin then injected was as effective as in normal guinea pigs. Kahn (96), more recently, has shown more precisely, by quantitative methods, the tendency for antitoxic serum to become bound to hypersensitive tissues and thus fail to be absorbed and exert its proper neutralizing action. Hooker and Follensby (97) have shown that, in human subjects markedly hypersensitive to horse serum, scarlet fever antitoxin, when injected locally, loses its neutralizing effect for specific toxin more quickly than it does in non-sensitive persons. They point out that it is quite probable that precipitins in the blood and tissues of these individuals may combine with antiserums and either remove them from the blood too quickly for them to exert an optimal therapeutic effect, or hold them at the area of injection and thus interfere with their effective absorption. The recent analysis of the variations in therapeutic results in patients treated with antiserums, by Davidsohn and Hunt (98) indicates quite definitely that, in patients sensitive to horse serum, and particularly in those giving accelerated reactions of serum sickness, the therapeutic results of serum administration were definitely poorer than in patients not sensitive to horse serum.

The intensified inflammatory reaction at the site of serum injection, besides interfering with adequate absorption, may, aside from the discomfort and pain, eventuate in severe or massive gangrene and even death. Several such cases have been reported within recent years (99, 100; 101, 102, 103), all in persons who had previously received horse serum injections. It is of interest that Arthus, in his first communication, called attention to the fact that these severe reactions might occur in humans if serums were given repeatedly. It is also important to note that, in the fatal case reported by Tumpeer and his associates, precipitins to horse serum were demonstrated in the blood of the patient.

Serum disease is another condition in which precipitins have been suspected of playing an important part. Hamburger and Moro (104) in 1903 found a definite relationship between serum disease and the

appearance of precipitins in the blood. They showed, also, that when horse serum was injected into rabbits it remained in the blood for about 8 days and then, with the appearance of precipitins, vanished from the blood. Von Pirquet and Schick (105) in the same year emphasized the probable importance of precipitins in serum disease and postulated an intracellular union of antigen and antibody as the origin of toxic materials which cause the symptoms of the disease. They called attention to the parallelism between the more rapid appearance of precipitins after reinjection of an antigen and the more rapid appearance of symptoms of serum disease after reinjection of horse serum. Later studies by Longcope and Rackemann (106) and Mackenzie and Leake (107) corroborated these opinions. They noticed in patients with serum disease, the frequent association of the early appearance of precipitins in the blood and severe manifestations of the disease. Furthermore, they observed that in those patients who escaped serum disease, precipitins could not be demonstrated in the serum at any time. Inasmuch as precipitins appeared after the development of symptoms, they concluded, as had von Pirquet and Schick, that the primary reaction of antigen and antibody was within cells, causing an explosive type of reaction whose severity depended upon the relative amounts of antigen and antibody available at different times. Tuft and Ramsdell (108) produced positive Prausnitz-Küstner reactions and passive anaphylactic sensitization of normal guinea pigs after injection of serum from patients with serum disease in more than half of the cases but were unable to find a similar correlation with precipitins by the methods used. Jones and Fleisher (109) and Khorazo (110), in their studies of experimental serum disease in the rabbit, found no correlation between the precipitative potencies of serums and the incidence of local reactions of so-called serum disease. It should be noted, however, that they used the antigen-dilution method for the determination of precipitin-titer.

Another possible factor in the development of serum sickness is the formation of heterophile antibodies (agglutinins and hemolysins for sheep's erythrocytes). Davidsohn (111) has found these agglutinins in the sera of all of 45 patients who had developed serum sickness, and in much higher titers than in patients who received horse serum but did not develop serum sickness. He concluded that "there seems to be a relation between the pre-existence of agglutinins for sheep cells before the injections of horse sera and the type of reaction. The rôle of the agglutinins may be merely that of an indicator of some change

predisposing for such type of reactions." At any rate, these findings constitute further evidence of the possible part played by flocculating antibodies in serum disease.

Do precipitins play a part in those intensified inflammatory reactions of infectious disease where edema, smooth muscle contraction, intensified inflammation, necrosis and reparative phenomena are so conspicuous? Do they also play a rôle in those hypersensitive conditions peculiar to man, the so-called atopic states, in which there is no evidence usually of previous sensitization, and where the hereditary predisposition is so striking? Are these conditions related to, or identical with anaphylaxis? It must be admitted at the outset that no answer can be given, as yet, to these questions, and there is certainly no proof that precipitins play a part in their development. Nevertheless, the fact that hypersensitive reactions may occur in the absence of demonstrable precipitins in the blood serum does not exclude the possibility that precipitins may be present within the cells. Methods now employed for the detection of precipitins, when present in low concentration in the blood and tissues, are crude and imperfect. Furthermore, it is reasonable to suppose that precipitates may range in size from tiny invisible molecular aggregates up to the larger precipitates seen under the usual conditions of precipitin determination. Variations in molecular size, either of antigen or antibody, may account for situations in which there is no definite evidence of antigen-antibody union in the test tube and yet a union can be demonstrated in living tissues by passive transfer of serum. Furthermore, the assumption of the existence of intracellular antibodies has long formed the basis of most of the current theories of anaphylaxis. Weil, in particular, believed that the answer to many immunologic problems must be sought within cells rather than in the blood and that the study of immunity is largely a problem of cellular physiology (112). In referring to the mechanism of anaphylaxis he said, "If, in place of the visible alteration, expressed as precipitation in the test-tube, interaction of the two factors *in vivo* is supposed to produce an alteration of cellular equilibrium, such as will act as cellular stimulus, all the requirements of the problem are satisfied. In view of the fact that precipitin has been demonstrated to be identical with the sensitizing antibody, this explanation of anaphylaxis seems almost self-evident" (113).

That the intracellular effect is really aggregative, however, has not been shown objectively, but there is little doubt about the fact that the intracellular union of antigen and antibody can occur directly and

can cause a profound change in cellular metabolism, apparently independent of circulatory and nervous mechanisms. For example, Holst (116), and Stewart and associates (117) have shown that tuberculin, added to leukocytes from animals hypersensitive to it, is directly toxic and leads to the death of the cells. Dienes and Mallory (116, 117) have shown that, in guinea pigs hypersensitive to tuberculin, necrosis of epidermis developed too early to be accounted for by a circulatory disturbance, indicating, therefore, that the antigen-antibody union occurs directly within the epidermal cells. Similar results have been obtained by the use of tissue cultures. Rich and Lewis (118) observed that cells from the blood and the fixed tissues of tuberculous animals, when grown in tissue culture, were distinctly more susceptible to the toxic action of tuberculin than were normal cells, due, they suggested, to the presence of specific antibody in or attached to the cells. Aronson (119) and Moen and Swift (120) have confirmed these findings. Aronson demonstrated that tissue culture explants from the spleen and bonemarrow of tuberculous guinea pigs were extremely sensitive to tuberculin but that similar explants from animals sensitive to horse serum were not adversely affected by the addition of horse serum to the explants. Aronson suggested that this might indicate a fundamental difference between the mechanisms responsible for hypersensitivity to tuberculin and to horse serum. Moen and Swift found that the hypersensitivity of tissue cultures to tuberculin persisted through several transplantations. They suggested, therefore, that this "represented a more or less permanent acquired characteristic impressed on the cell as a result of the infection." They state that when tuberculin was added to the sensitive cells "various grades of coarse granulation and vacuolization of the cytoplasm developed; protoplasmic processes shortened . . . and cellular disorganization was followed by disintegration." This description suggests a process of coagulation necrosis, due, possibly, to intracellular precipitation. Moen later reported (121) a similar effect in suspensions of mononuclear cells obtained from the pleural cavities of tuberculous animals; he noted that the sensitivity to tuberculin persisted through three transplantations and over a period of 29 days.

The differences in sensitivity to such substances as tuberculin, horse serum, egg white, etc., of cells in tissue culture, do not mean, necessarily, that the different reactions of hypersensitivity are fundamentally different. These proteins vary greatly in molecular size, and presumably, therefore, in diffusibility. Neurath (122) has recently given

molecular weights for ovalbumin as 40,500, for horse serum albumin as 67,000 and horse globulin, 150,000. Seibert and associates (123) have also shown, by similar methods of determination of molecular weights, that those for fractions of tuberculin protein may vary from 32,000 to 10,000. It is possible, therefore, that some of the smaller active molecules may diffuse into hypersensitive cells in tissue culture when larger molecules cannot. It should be remembered that Seibert showed (124), some years ago, despite many statements to the contrary, that the Arthus reaction could be elicited in rabbits and guinea pigs by the injection of tuberculin protein of larger molecular size but that fractions with smaller molecules had a haptene-like action and gave a sharp "delayed" cutaneous reaction but could not serve as sensitizing antigens to produce the Arthus phenomenon. It may be, therefore, that the differences in reactivity of sensitized cells upon contact with proteins of such varying molecular size and diffusibility are more apparent than real.

If it could be shown that the cellular reactions of atopy are analogous to those of anaphylaxis, therapeutic procedures might be more clearly formulated but, despite the great amount of study of the problem of pollen sensitization alone, its underlying mechanism is not well understood. Although the antibody (reagin) can be transferred passively to the skin of a normal individual, precipitins cannot usually be demonstrated in the serum, at least not by the methods now available (125, 126). Nevertheless, in experimental animals, injection of pollens engenders the formation of specific precipitins (127, 128) and these antiserums may induce passive sensitization in man. Furthermore, "neutralization" of these serums with atopen removes the skin-sensitizing property (129). It may be that the molecular structures of atopen and reagin prevent the formation of visible aggregates in many instances but that invisible aggregation, within a cell, may cause functional impairment and the resulting edema and wheal formation. It is also possible that atopic individuals may possess tissues which vary in their permeability to reagins and atopens. The experiments of Cohen, Ecker, Breitbart and Rudolph (130) demonstrated a marked delay in the absorption of ragweed pollen after its insufflation into the nostrils of atopic individuals, indicating that such persons have "developed a mechanism for the partial exclusion of foreign materials from the blood stream and the tissues."

Limitation of space prevents the consideration of many other pathological conditions in which tissue injury has been ascribed to a combi-

nation of antigen and antibody within cells. Many of the exudative, degenerative and reparative tissue reactions seen so commonly in tuberculosis, lobar pneumonia, streptococcic infections, parasitic infections and in rheumatic disease, necrotizing arteritis, periarteritis nodosa, Libman-Sacks disease, glomerulonephritis, arthritis, asthma, serum carditis, serum neuritis etc., have been thought to be examples of allergic inflammation. This phase of the subject has been reviewed recently by Opie (65) and by Stenn (131). It is apparent, however, that too little is known as yet about the nature of these reactions to justify a definite opinion about the causative relationship of allergic inflammation to the pathogenesis of these conditions. The present uncertainty but emphasizes the imperative need of more basic information about antigen-antibody reactions in various types of tissues.

SUMMARY

This discussion, although necessarily limited in scope, points definitely to the importance of agglomerating antibodies in tissues and indicates the need of greater emphasis on their earliest effects. Facts have been recorded demonstrating that agglutinins may combine with their respective antigens both in the blood and tissues and thereby promote adherence of the microörganisms to one another and to the tissues. This process, by its opsonizing effect, also promotes phagocytosis and the more effective localization and destruction of the invading agents. Precipitins, similarly, have been shown to react in the blood and tissues, causing either an accelerated removal of antigen from the blood or its prompt localization near the portal of entry. The evidence strengthens the view that these agglomerating reactions may be looked upon, fundamentally, as part of the mechanism whereby foreign proteins which may enter the tissues parenterally, are immobilized and destroyed, thus tending to ensure the integrity of the body's proteins. The adverse effects may be considered, essentially, as toxicological byproducts of the reaction, depending presumably upon quantitative variations in kinds and relative amounts of antibody and antigen reacting; under some circumstances these reactions may constitute actual disease, as with serum disease and allergic inflammation from various causes. It is not surprising that antigenic stimuli which, when minimal, cause insignificant tissue reactions may, in an exaggerated form, lead to profound cellular injury or even death; this does not mean that the mechanism itself is necessarily at fault. Cellular integrity must be maintained, presumably, by mechanisms inherent in the cells themselves,

and any condition, whether natural or artificial, which interferes with these may be expected to be reflected in cellular disturbances which may, at times, be definitely pathological. The fact remains that these effects (edema, smooth muscle contraction, intensified inflammation, necrosis, and reparative sequelae) all follow cellular stimuli; it would seem more logical, therefore, to place the blame for the adverse reactions on the stimulus rather than on the cell. This point of view recognizes, at least, that although a biological process may at times be harmful to the individual, yet, in its more fundamental aspects, it may be protective to the race.

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FUNCTIONS OF THE CAROTID AND AORTIC BODIES

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The carotid and aortic bodies are parts of the system of cell groups, variously called chromaffine bodies, paraganglia, glands, nodules, and glomera, which have long been known by anatomists to be constantly present in intimate association with major arteries but concerning whose function nothing definite was known until the discovery of carotid sinus reflexes opened possibilities previously unsuspected. The major if not sole function of the carotid bodies (certainly) and aortic bodies (very probably) is now known to be due to the presence within them of chemoreceptors (or chemoceptors), i.e., nerve endings specialized to respond to certain changes in the chemical composition of their environment (which is arterial blood), such changes giving rise to reflexes which can produce physiologically important effects upon respiration and circulation. The most effective stimuli to the chemoreceptors are asphyxia, anoxia (produced either by reduced oxygen pressure in the arterial blood or by poisons, such as cyanide, which inhibit cell oxidations), acidosis, and drugs having "nicotinic" properties; they are also stimulated by hypercarbia.

I. Anatomical. For the literature on these aspects of the subject see the papers by Nonidez (59, 60), Boyd (10), Heymans, Bouckaert and Regniers (47). Ask-Upmark's monograph (3) is admirable for the comparative anatomy. In briefest summary, the points of greatest importance to physiologists are as follows:—*Embryologically*, the carotid body has a dual origin from the mesoderm of the third branchial arch artery and the ectoderm of the glossopharyngeal nerve, and it develops in the region in which these two tissues come into earliest juxtaposition; elements from the sympathetic and the vagus enter only at a comparatively late stage (Boyd). The aortic body presumably has a similar origin from the fourth left branchial arch artery and the vagus nerve, but satisfactory studies are peculiarly difficult to make in this region and the relationships are not as clear as they are in the third arch (Boyd). Recent workers are agreed that the carotid and aortic bodies are not "chromaffine" in the usual sense (DeCastro, Nonidez, Boyd), which is perhaps further evidence against their being intimately related to the sympathetic nervous system. The structure of these bodies is unusual in that the afferent arteries open directly into sinusoidal spaces lined only with endothelium and peculiar "glomus cells," the latter being very richly supplied

with nerve endings (DeCastro); the abundance of nerve elements, separated by the thinnest of walls from vascular spaces in which the pressure must be close to the arterial level, is a morphological characteristic of greatest significance to physiologists.

The blood-supply of the carotid body is by way of a small artery which arises from the external carotid or one of its major branches (occipital in the dog); the afferent artery does not terminate in the carotid body but passes on to give off branches to the pharyngeal and prevertebral regions, where the vessel anastomoses freely with other carotid (and vertebral) branches—a fact of importance in physiological experiments (76, 16). In the aortic region the situation is more complicated because of the presence of several groups of tissue of this general type (59, 60, 10), supplied with arteries which may arise variously from the brachiocephalic, aorta, coronary artery, and pulmonary artery. The recent work of Comroe (15) strongly suggests that only one of these bodies retains functional significance in the adult animal, and this is supplied by a branch arising from the transverse aorta in the dog, from the coronary arterial system in the cat.

The nerve fibers from the carotid bodies enter the central nervous system with the sinus (or intercarotid) branch of the glossopharyngeal. Fibers can also be traced from this region to the sympathetic and vagus ganglia in the vicinity, but the functional significance of these is uncertain; it is generally agreed that carotid chemoreceptor functions are completely abolished by section of the sinus nerve. The nerves from the aortic body of the dog join the vagodepressor trunk in the region of emergence of the recurrent laryngeal nerve (on the right side certainly, on the left probably); the afferent nerve from the aortic body can sometimes be isolated on the right side and identified by the physiological effects of its stimulation (15). Impulses from the aortic body (or bodies) are however carried by both vagodepressor nerves, for section of one of these may reduce but it does not abolish the activity of the aortic chemoreceptors.

Localization of the chemoreceptors in the carotid and aortic bodies. Since the *carotid body* of the dog is usually located on the occipital artery, which typically arises at a distance of 2–3 mm. from the carotid sinus proper, it is possible in this animal to demonstrate clearly that the carotid reflex mechanism is a dual one. The first to do this successfully were Bouckaert, Dautrebande, and Heymans (8), who inactivated the nerve fibers leaving the carotid sinuses by tying ligatures about them; they found that this removed the response to pressure changes (hypertension on carotid occlusion) without abolishing the effects of chemical excitants. Camus, Bénard, and Merklen (14) obtained the same result by actual section of the fibers from the pressoreceptors. Heymans and Bouckaert (44) also performed the complementary experiment, in which activity of the pressoreceptors was retained after the chemoreceptors had been inactivated by embolization of their blood-vessels with lycopodium spores. The latter experiment does not, of course, prove that the chemoreceptors are located in the carotid body. Evidence in that direction was furnished by Schmidt (64), who found that ligation of the occipital artery at its origin in dogs reduced or abolished the reflex effects of alterations in gas content of the blood in the carotids, and by Gollwitzer-Meier (39), who obtained similar results with respect to injections of lobeline into the carotid of the dog, but neither of these workers could decide whether the chemoreceptors were located in the carotid body or in the first portion of the occipital

artery. DeBettencourt and Cardoso (20) confirmed most of the above-cited observations and made some additional ones which led them to conclude that the carotid body is probably the most important point of origin of chemically aroused reflexes, though it may not be the sole one. Comroe and Schmidt (16) showed that the effects of intracarotid injections of small doses of lobeline or cyanide were entirely abolished on clamping the small artery to the carotid body and restored on opening this vessel; this showed that the carotid body is the only location of importance for chemoreceptors in this region. They also concluded, from vessel anastomosis and perfusion experiments, that the pressoreceptors are limited to the carotid sinus region and are not present in the occipital artery or carotid body. The latter conclusion is probably sound for all practical purposes, but recent experiments (unpublished) on dogs have indicated that some pressoreceptors are invariably demonstrable (by action potentials) in any preparation in which chemical sensitivity is retained, even after complete stripping of the fibers running from the carotid sinus to the carotid body, denudation of the external carotid, and firm ligation of this vessel between internal carotid and occipital. The number of these receptors must be small and their physiological importance questionable. Perhaps there are also some chemoreceptors in the pressoreceptor area of the carotid sinus; if so, their presence has not been revealed by the methods so far used to detect it (13, 20, 16). At present it seems probable that true chemoreceptors are sharply localized in the carotid body; pressoreceptors are certainly scattered more widely, and perhaps DeCastro (22) was quite correct in his belief that some of the nerve elements in the walls of the arteries in and near the carotid body have the same function as the similar structures in the carotid sinuses.

Localization of the *aortic* chemoreceptors in the *aortic body* rests upon the anatomical similarities between this and the carotid body (60) and upon the physiological experiments of Comroe (15). The latter was able, by intraaortic injections of cyanide or lobeline through a small catheter which could readily be moved in and out, to demonstrate that the chemosensitive zone lies in dogs at a point at which a small artery leaves the aorta to enter the aortic body. Furthermore, he could (in favorable subjects) identify a nerve leading from this region to the vagodepressor trunk, electrical stimulation of which produced effects exactly like those elicited by chemical excitants. In one experiment he was able, by crushing the nerves in the vicinity of the aortic body, to abolish chemoreceptor activity in the aorta while retaining at least some pressoreceptor function. Thus the anatomical as well as functional separation of the two sets of receptors has been accomplished for the aortic as well as carotid zones of the dog.

Other chemoreceptor tissues. Nonidez (59, 60) and Boyd (10) both were able to detect several other groups of glomus-like tissue in the region of the aorta and pulmonary artery, and Nonidez (60) found that the structure corresponding with the aortic body in Comroe's experiments received blood from the pulmonary artery in new-born cats. Comroe was unable to find any physiological evidence of this relationship in adult cats or dogs; apparently the communication with the pulmonary system closes some time after birth, but the functional significance of this, or of the anomaly that would result if closure did not occur, has not been investigated as yet.

Other groups of tissue morphologically similar to the carotid and aortic bodies (such as the coccygeal body) have not been investigated from the standpoint of

chemoreceptor function.. In many if not most cases denervation of the carotid and aortic bodies practically abolishes the stimulant response of respiration and circulation to anoxia, however produced, but there are some instances (see p. 121) in which a more or less distinct response persists. Perhaps in these, unusually competent masses of similar chemoreceptors are located elsewhere. On the other hand, there is no reason why all chemoreceptors should affect the same functions; perhaps other groups produce responses totally different from those now under consideration. Evidence on these points is lacking.

II. Physiological properties of the carotid and aortic bodies. These will be taken up, in the order of the historical development of this subject, first from the qualitative and next from the quantitative viewpoint.

A. Qualitative. 1. The nature of the response. Increased activity of the chemoreceptors of carotids or aorta leads to increased activity of the respiratory and vasomotor centers (in the case of the carotid chemoreceptors, of the cardioinhibitory center also), and decreased activity elicits opposite effects. On this point there is no longer any room for doubt: the observations first made by J. F. and C. Heymans (49) on the aortic region and by C. Heymans, Bouckaert, and Dautrebande (46) on the carotid reflex zone have been confirmed by a large number of different workers on a variety of animals and by a variety of technics. The pertinent literature has been cited recently by Heymans and Bouckaert (45), Gesell (36), and Gellhorn and Lambert (32), and will be presented later in this paper. The distribution of the effects arising in the chemoreceptors is the same as that related to the pressoreceptor reflexes but the direction of the effects from the two types of receptors is exactly opposite (with the exception of cardioinhibitory activity, which is definitely increased by increased discharge from either the pressure- or chemo-sensitive receptors of the carotids). That the stimulation of respiration and blood-pressure arising from the chemoreceptors is due to a positive stimulant effect by the nerve impulses upon the centers and not (like stimulant effects referable to the pressoreceptors) to a removal of an inhibitory influence, was shown by the occurrence of hyperpnea and hypertension when a nerve joining the aortic body to the right vagodepressor of the dog was stimulated electrically (15); the same conclusion may also be derived from studies of the electrical activity believed to arise in the carotid chemoreceptors (50, 7, 88, 63, 29). These will be considered in a subsequent section of this paper (p. 141).

2. The nature of the stimulus. There is also no good reason for doubt on the qualitative aspects of this question. Leaving out of account for

the present special stimulants such as acetyl choline and potassium (see p. 149), it is safe to say that all who have done enough work in this field to acquire the requisite technic have been able to satisfy themselves of the general truth of the conclusions of Heymans et al. (46, 47), namely, that the chemoreceptors can be stimulated by each of the three chemical agents that have long been known to be capable of stimulating respiration and circulation, i.e., fall in pH, rise in the tension of CO_2, and anoxemia. Furthermore, there is general agreement that, as far as anoxemia is concerned, the stimulant effects on respiration and circulation are due much more to these reflexes than to anything else, and a number of workers believe that reflexes are solely responsible for such effects. The latter opinion is not universally held, however, even for anoxemia, and there never has been any serious claim that these reflexes are exclusively, or even dominantly responsible for the reactions of the whole organism to the changes in chemical composition of the blood associated with ordinary physiological activities. Consequently the assessment of the function of the chemoreceptors involves quantitative considerations such as relative sensitivities, speeds, intensities, and durabilities of the chemoreflex elements, on the one hand, of the nerve cells of the centers on the other.[1] We propose, in the following pages, to attempt to evaluate the existing evidence on these points from as objective a viewpoint as is possible to us, devoting more attention to the experimental evidence upon which existing ideas are based than to the conclusions drawn by the various authors, for the latter have been reviewed elsewhere (47, 45, 36, 32).

B. Quantitative. 1. Anoxemia. Of all quantitative aspects of the subject this is in the most satisfactory state, for, as already pointed out, all agree that the stimulant effects of anoxemia upon respiration and circulation are much more reflex than central. Since the ensuing discussion is to deal with the validity of experimental evidence, this is a good place to point out the nature of that evidence.

The development of knowledge on the quantitative aspects of this problem began with the work of Heymans, Bouckaert and Dautrebande (46), and their experiments bearing on these points were of two main types. One was the relatively simple procedure of testing the

[1] For the purposes of this review we shall assume that all of the non-reflex responses are due to direct effects upon the centers, though we realize that this is not necessarily the case: other chemoreflex mechanisms, as yet unidentified, immediately come to mind, but we see no point in adding further complexity to an already complex situation by speculations along those lines.

response of the intact animal (they used dogs under chloralose anesthesia in nearly all of their work) to a given stimulus (in this case anoxemia produced by inhalation of nitrogen or hydrogen), and repeating the test after section of the sinus and depressor nerves. The other was the more complicated procedure of preparing one or both carotid reflex zones, by ligation of vascular branches while leaving the innervation intact, so that this region could be perfused, either by means of a pump or by anastomoses with the blood-vessels of a donor animal, with fluid whose chemical composition could be varied at will, independently of the blood reaching the medullary centers. These two types of experiment have been used, with variations in detail but not in principle, by most workers who have subsequently entered this field.

As far as anoxemia is concerned the results of both sets of experiments have agreed very well. Heymans et al. found (and presented an impressive example as evidence) that section of the sinus and depressor nerves changed the violent hyperpnea and marked hypertension produced by nitrogen inhalation into a very slight respiratory stimulation and a relatively small rise in blood-pressure. In their crossed-circulation experiments they showed that anoxemia (from nitrogen inhalation) in the donor animal can cause strong reflex hyperpnea and hypertension in the recipient. The simple denervation experiment has been repeated by a number of workers (6, 71, 64, 33, 34, 83, 84, 86, 75, 38, 26, 43, 58, 11, 32, 73, 16, 15) and the result confirmed in the main, for cats and rabbits as well as dogs, and for the unanesthetized or decerebrate animal as well as the narcotized one. The perfusion experiments also have been repeated (64, 31, 28, 4, 5, 77, 16) and, in so far as pertinent observations were made, results entirely confirmatory of those cited above were obtained: the respiratory and (under favorable circumstances) vasomotor centers were shown to be reflexly stimulated by reduction in oxygen pressure in the carotid blood.

Questions concerning the relative *sensitivity, speed, intensity, and durability* of the reflex and non-reflex components of the anoxemic response are comparatively easy to answer because there seems no good reason to doubt that the reflex system is much the more sensitive, rapid, powerful, and resistant. It is true that a number of workers (71, 64, 58, 15) found that denervation of the carotids alone did not entirely remove (though it usually greatly reduced) the hyperpnea of anoxemia, but in these cases the depressor nerves were intact, and when the test was repeated after they were cut the stimulant response was gone (71, 64, 15). This is, of course, in entire accord with the work

of J. F. and C. Heymans (49), indicating that similar chemoreceptors are located in the aortic region. But instances are not lacking (18, 23, 36) of a distinct anoxemic hyperpnea remaining after section of the sinus and depressor nerves, which may mean, as Gesell (36) states, that the nerve cells of the respiratory center are capable of being stimulated by anoxia. This is certainly not improbable; the remarkable feature is that, in the light of available evidence, the response of these nerve cells is as slight and uncertain as it appears to be. As Wright points out (71): "Too much significance must not be attached to the stimulation sometimes observed with very severe anoxaemia (in animals deprived of the afferent impulses from the sinuses and vagi), since one is then dealing with a centre on the verge of death." The contrary opinion voiced by Dautrebande (18), who claimed that anoxemia (due to high altitudes) still causes distinct hyperpnea in unanesthetized dogs whose sinus and depressor nerves had been severed some time previously, need not be given undue weight for the following reasons: he did not actually determine the presence of hyperpnea, but concluded that it must be present because arterial CO_2 content was reduced; even if there was hyperpnea, its relation to anoxemia remains uncertain in view of the circumstances (unanesthetized dogs taken on a journey, subjected to strange surroundings, perhaps to cold and to other factors capable of affecting breathing); the denervation may have been incomplete, either from the start (chronic hypertension does not of itself prove denervation of the chemoreceptors, for the latter may remain active, in carotids and aorta, after the fibers from the pressoreceptors have been cut), or because of regeneration; section of the depressor nerves—uncertain at best in dogs—does not interrupt all of the fibers involved in aortic reflexes, for some of these are carried by the vagus (54). Bouckaert, Heymans, and Samaan (9) recently found, in unanesthetized dogs with vagi cut and sinuses denervated, no sign of the hyperpnea postulated by Dautrebande.

The available evidence strongly indicates that the *threshold* of the chemoreceptors to anoxemia is decidedly lower than that of the center. Granting this, the best way to ascertain the value of that threshold is simply to determine the smallest degree of anoxemia to which the whole organism reacts with hyperpnea. The lowest acceptable value of which we are aware is that given by Ellis (24) for normal men, namely, respiratory stimulation at a decrease to 18 per cent oxygen in the inspired air. This corresponds with a diminution of about 23 mm. in the oxygen tension of the inspired air (assuming O_2 = 21 per cent of

760 mm. Hg atmospheric pressure in the control period) and therefore with a resulting arterial oxygen tension of 67 mm. (assuming this to be 90 mm. in the control period). This may be regarded as the highest sensitivity to anoxemia that one has any right to expect from the chemoreceptors.

There are a few corresponding direct observations on animals, made in the course of perfusions of the carotid bodies with blood, and while they are not entirely satisfactory they agree reasonably well with the figure just cited for normal man. Schmidt (64) found a diminution of about 5 vols. per cent in the oxygen content of blood in the carotids of a decerebrate cat when reflex hyperpnea was well under way, and in a similar experiment on an anesthetized dog a change of about the same size was found present; data for calculation of arterial oxygen saturation were not obtained, but since the donor animals were breathing oxygen during the control period these bloods (which were pumped directly from the arteries of the donors) probably were almost saturated with oxygen. If we assume 100 per cent saturation beforehand, the response in the cat began at a saturation of about 64 per cent, that of the dog at about 70 per cent. Taking (for purposes of illustration) the dissociation curve of human blood at 40 mm. CO_2 pressure (Haldane, p. 71), these figures would represent a reduction to about 35 mm. O_2 pressure in the cat, to about 40 mm. in the dog. In the later experiments of Comroe and Schmidt (16) reflex hyperpnea was found to begin at a decrease of about 4 vols. per cent in arterial oxygen content; here again oxygen inhalation was used during the control period, which means that the blood was probably about 80 per cent saturated at a 4 per cent diminution in O_2 content and the arterial oxygen pressure under the latter conditions would work out at a little less than 50 mm. Bernthal (4) found carotid reflex responses beginning with blood equilibrated against 15 per cent oxygen; if we assume—as above—that the blood was saturated when equilibrated against the 18 per cent oxygen used in the control period, the response began at about 83 per cent saturation or about 52 mm. oxygen pressure. If the bloods were not 100 per cent saturated with oxygen in the control period, these values would be lowered correspondingly.

With regard to the *rapidity* with which the chemoreceptors and the center respond to anoxemia, there is again quite general agreement that the former are vastly superior. Heymans, Bouckaert, and Dautrebande (46), in their published example of the influence of sinus and aortic denervation, showed the hyperpnea and hypertension to be not

only greatly diminished but also considerably delayed after the denervation. Gemmill, Geiling and Reeves (33) and Henderson and Greenberg (43) obtained similar results after sinus denervation alone. Selladurai and Wright (71), however, found that in denervated decerebrate cats breathing might sometimes show an initial great acceleration upon the induction of anoxemia, but they interpreted this as one manifestation of derangement of the central mechanism by anoxemia when the protective chemoreflexes are removed. It is practically certain that the vigorous hyperpnea which arises promptly when acute anoxemia is induced is due to stimulation of chemoreceptors rather than center.

The relative *strengths* of the reflex and central components of the anoxemic response are also quite clear. Nobody has claimed that the center is able to match the vigor of the chemoreceptor response; all agree that after denervation of the chemoreceptors any anoxemic hyperpnea is relatively weak. Perfusion experiments have also shown that the reflex responses to anoxemia rival in intensity those expected (or actually elicited) when the whole organism is exposed. This was strongly suggested by the experiments of Heymans, Bouckaert, and Dautrebande (46) and of Schmidt (64). It was actually shown to be the case in the experiments of Comroe and Schmidt (16), who found that the hyperpnea produced by inhalation of nitrous oxide by an animal with one carotid body functioning was equalled by that elicited by perfusion of the same blood through the other carotid body of the same animal; if neither carotid body was functioning during the inhalation, there was no appreciable hyperpnea, but blood collected at that time caused vigorous hyperpnea when subsequently perfused through a carotid body.

Finally, with regard to the relative *durability* or resistance of the chemoreceptors and the center when exposed to anoxemia, there seems no doubt that the former are again greatly superior. The fact (46, 64, 71, 84, 87, 33, 34, 26, 73, 32, 15, 16) that severe anoxemia may cause pure respiratory depression or failure when the chemoreceptors are denervated indicates that the "wreckage of machinery" (Haldane) that anoxia has for many years been known to exert upon actively metabolizing tissues becomes evident in the nerve cells of the respiratory center under such circumstances. Frequently there is a fall in blood pressure when respiration is failing or has just failed at these times, and Gellhorn and Lambert (32) attribute this to extreme depression of the vasomotor center. While this is, of course, highly probable, the reviewers do not agree with them in believing that cardiac depression can be excluded

as a factor of considerable importance. By contrast with the depression and failure of respiration and circulation that are typical results of acute anoxemia in the absence of chemoreceptor activity, the persistence of respiratory activity in the presence of comparable anoxemia with the receptors functioning indicates that the latter are much more resistant than the centers to the depressant effects of anoxia.

Intimately related to anoxemia are the actions of *cyanides and sulfides,* and the effects of these on the chemoreceptors are entirely comparable with those of anoxemia. This was first shown by Owen and Gesell (61), and has been abundantly confirmed since (see Gesell, 36). As with anoxemia, there is some evidence (see 36) to indicate that the centers may be directly stimulated, but for this considerably larger doses are required than those that suffice to stimulate the chemoreceptors, and the central stimulation is certainly much the weaker factor. Large amounts can depress the centers, but a depressant effect on the chemoreceptors, although it probably exists, has not yet been demonstrated. Thus the extraordinary resistance of the chemoreceptors to the disorganizing effects of anoxia is again conspicuous. Other drugs—notably nicotine and lobeline—act like cyanides and sulfides in these respects (see p. 147).

An interesting quantitative difference between anoxemia and cyanide (or lobeline) as a chemoreceptor stimulant has been pointed out by Gesell (36), who states that in recent experiments (unpublished) in his laboratory cyanide has been found to be a distinctly stronger reflex stimulant than anoxemia (produced by decreased oxygen tension in the arterial blood). He suggests that this may be due to the decreased CO_2 tension and hydrion concentration that would result from diminution in the oxygen content of the blood, the anoxic stimulus in the chemoreceptors thus being opposed by simultaneous reduction in the other two stimuli. We have made similar observations, but have found the same relationships to hold when a saline fluid was used instead of blood for perfusion of the carotid bodies. In such cases there must be another explanation for the superiority of cyanide to anoxemia (produced by saturating the perfusion fluid with nitrogen or nitrous oxide). It may be that the anoxia produced within the receptors by a poison which—like cyanide—strikes at the intracellular activation of molecular oxygen, is more rapid in development and greater in degree than that resulting simply from reduction in the pressure of molecular oxygen in the blood. Both factors would probably operate under ordinary conditions.

2. Carbon dioxide. The actual experimental evidence on the quantitative aspects of this problem is fairly consistent though the conclusions drawn from it are not. Heymans, Bouckaert, and Dautrebande (46) showed a record in which the pneumographic response of a dog to inhalation of 3 per cent CO_2 was not significantly different whether the sinus and aortic nerves were intact or cut. In reporting this result they stated (l.c., p. 428):—"L'action stimulante respiratoire centrale du CO_2

se superpose donc à l'action stimulante réflex (d'origine sino-carotidienne et cardio-aortique)." This simple experiment has also been repeated by a number of different workers, and nobody (as far as the reviewers are aware) has failed to make substantially the same observations as those just indicated. The question here is not, as in the case of anoxemia, whether any response remains after the denervation, but whether the response has been affected significantly. Among those who have reported such experiments, Selladurai and Wright (71—anesthetized and decerebrate cats), Schmidt (64—anesthetized dogs and cats), Green and DeGroat (40—unanesthetized dogs), and Euler and Liljestrand (26—anesthetized cats) mentioned more or less diminution in the respiratory response to CO_2 after the denervation whereas Schmidt (64—anesthetized rabbits), Wright (84, 86—anesthetized and unanesthetized rabbits), Stella (75—anesthetized dogs), Gemmill and Reeves (34—unanesthetized dogs), Gesell and Moyer (38—anesthetized dogs), and Smyth (73—unanesthetized rabbits) found either that the result was practically unaltered, or else did not comment on the relationship beyond stating that a strong response remained after the denervation.

At first glance these results appear to represent completely random scattering, which may be true, but it should be noted that with only one exception (Green and DeGroat) all who have used unanesthetized animals have found no significant effect by the denervation on the CO_2 response, and the experiments of Green and DeGroat are open to several criticisms. In the first place, although they used three denervated dogs, they tested the CO_2 response before the denervation in only one, so that their experiment is really limited to a single animal. Second, their procedure was such that the rate of increase in alveolar CO_2 tension depended on the vigor of the animal's breathing when the inhalation began, and it is only reasonable to expect that the oftener a dog is used for such experiments the calmer he will become; their conclusions are based largely on the fact that the respiratory response after the denervation began later and progressed more slowly at first but to ascribe this to the denervation is scarcely justified. Selladurai and Wright (71) noted that the effects of the denervation were less consistent and less marked in decerebrate cats than in anesthetized animals. The rabbit's response to CO_2 appears not to be affected by the denervation, whether the animal is anesthetized or not.

If—as seems probable—the main factor in these discrepancies is anesthesia (perhaps abetted by trauma, irritation of afferent nerves, etc.), it is clear that they can have no decisive bearing on the question under

consideration. There is evidence (56, 16) that under anesthesia chemoreceptor reflexes play a larger part in respiratory activity than is the case in the intact animal. The positive results enumerated above may be (probably are) only further evidence in that direction and not proof that the response of the intact normal animal to CO_2 is measurably dependent upon chemoreceptor activity. In any case, the consistently negative outcome of similar experiments in rabbits would preclude generalizations. One is justified in concluding from the denervation experiment that the contribution of the chemoreceptors to the hyperpnea of hypercarbia is certainly less important than the contribution of the center itself, and much less important to the total effect than the reflex component of the hyperpnea of anoxemia.

These conclusions are supported by the published data obtained in perfusion experiments. Heymans, Bouckaert, and Dautrebande (46), as proof of the great sensitivity of the chemoreceptors to changes in CO_2 tension, cite an experiment in which both carotids of a dog were perfused with Ringer's fluid containing variable amounts of CO_2 and buffered to constant pH. In this experiment respiration was reflexly stimulated when a fluid containing 7.4 vols. per cent CO_2 at pH 7.4, was replaced with one containing 36.3 vols. per cent CO_2 (pH 7.4); a further change to a fluid of 43.6 vols. per cent CO_2 (pH 7.4) caused further (though slight) respiratory stimulation, and finally a change to a fluid of 50.4 vols. per cent CO_2 at pH 7.9 caused respiratory depression. The quantitative value of this evidence cannot be determined from the available facts because nothing is said about the temperature at which the equilibrations and pH estimations were made, nor whether gas was allowed to escape between equilibration and entrance of the fluid into the carotids if the fluid was warmed after equilibration. Without information on these points the CO_2 tension, which must have been the determining influence, cannot be estimated with any accuracy. If we take the above-mentioned figures as they stand, however, the CO_2 tension of the first solution would be about 5 mm. Hg, of the second about 25, of the third about 30, and of the fourth approximately 12—all at 38°. Apart from the change from the 25 to the 30 mm. solution, the magnitude of the variations in CO_2 tension required to produce a reflex respiratory effect (which was far from marked) was not such as to indicate great sensitivity. The change from 25 to 30 mm. is, as they state, about that to be expected between arterial and venous blood. Since the same example is reproduced in the recent review by Heymans and Bouckaert (45), it seems fair to assume that this is the

best result obtained by them. If that is true, one is entitled to conclude that the threshold of the carotid chemoreceptors was never less than 5 mm. under the conditions of their experiments, and that result has recently been duplicated in a series of similar experiments on dogs now under way in this laboratory (66); in these, however, the 5 mm. threshold was encountered only once in 5 very reactive animals, a 10 mm. change being the minimum required for some, a 15 to 20 mm. change for others.

These results of Heymans et al. have been widely quoted as proof of the very great sensitivity of the chemoreceptors to changes in the CO_2 tension of the blood although, as noted above, no actual data bearing on that point are given in their paper. Even if they had been, one might still hesitate to accept them without some indication of the number of experiments performed and of the frequency with which this result was obtained. To draw a conclusion as important as this from the result of one experiment is to ignore the factor of individual variability—which it is never safe to do in a biological investigation, and which is particularly unjustifiable here because Comroe (15) has been able to show wide individual variations in the effectiveness of chemoreceptor reflexes. Furthermore, as the reviewers know from personal experience, there is a psychological hazard in experiments like this which can easily lead to exaggeration of the value of an unusually favorable result, in the following manner: the preparation involves extensive dissection, ligation of vessels, artificial circulation, and numerous other factors, all operating in the direction of diminished effectiveness of the reflexes. The experimenter must be prepared to find that a certain proportion of such preparations will have inactive reflexes, and this he will (quite properly) ascribe to artifacts. In another (and larger) group, reflexes will be present, though of variable activity, and in a third (perhaps quite small) group they will be extremely active. It is natural to regard the most striking results as those to be expected in the absence of artifacts and to publish them as the closest approach to the normal state. Actually, however, the investigator can never know that to be the case; perhaps he was dealing with responses that are wholly exceptional, and, for all that he can tell, the less impressive results may really be closer to those to be expected in the average animal. Furthermore, as the experimenter's experience and skill increase he incorporates in his technic various items and procedures which he has found helpful to bring out the desired result in maximum intensity; in so doing he may remove the experimental conditions further and further from the normal state, but it is not easy to allow for this in drawing conclusions from experiments that represent a real masterpiece of technical proficiency. As an example of a factor of this sort the anesthetic (chloralose) used by Heymans in most of his work may be cited. The statement has been made elsewhere (65) that this drug exaggerates the pressure reflexes from the carotid sinus, and in recent experiments (68) on decerebrate dogs the same thing has been found true of the chemoreflexes, for chloralose consistently lowered the threshold of the carotid bodies to intraarterial injections of minute doses of cyanide. An additional example is the use of vagotomy and artificial respiration to reinforce the vasomotor reflexes from the carotid bodies.

It appears to the reviewers that the lesson to be derived from all this is that when the results of technically sound simple experiments conflict with those of comparably sound complicated ones, the burden of proof should be on the latter. In other branches of biology this is almost universally the case, but in this particular field there has been a notable tendency to glorify the complicated experiment. Specifically, their simple denervation experiment gave little justification for the belief that chemoreceptor reflexes play a prominent part in the organism's response to CO_2, because there was vigorous hyperpnea before and after denervation and the respiratory record was not quantitative, yet in their first paper Heymans, Bouckaert, and Dautrebande (46) concluded (p. 447): "La sensibilité réflexogène respiratoire des sinus carotidiens domine la sensibilité directe du centre respiratoire aux différent excitants: pression artérielle, ion hydrogène, CO_2 et anoxémie."

These perfusion experiments were repeated by Schmidt (64) and the results were confirmed qualitatively, not only in anesthetized dogs but also in decerebrate cats. By comparison with the effects of anoxemia, however, those due to simple hypercarbia seemed very slight; in one of the examples published (64—fig. 14), a reflex hyperpnea due to anoxemia in the donor was abolished just as promptly when (by mistake) the donor was given 10 per cent CO_2 in oxygen to inhale as had been the case when pure oxygen was given. Nevertheless definite confirmation was secured of the ability of the carotid chemoreceptors to respond to changes in CO_2 content of the blood, and in the example shown (64—fig. 15) an increase of 4.6 vols. per cent in arterial CO_2 content sufficed to elicit reflex hyperpnea in a dog.

Gayet, Bennati, and Quivy (31) also repeated the perfusion experiment on dogs, using defibrinated dog or beef blood diluted with an equal volume of Locke's fluid for the purpose. They reported marked reflex hyperpnea from increases in arterial CO_2 within physiological limits, which, in the example cited, is stated to be "une augmentation de 16 per cent de CO_2 dans le liquide artificiel." Whether this means an increase amounting to 16 vols. per cent (which seems probable, since CO_2 content was determined by Van Slyke's method) or a 16 per cent increase above the preëxisting level of blood CO_2, is obscure, but in either event the rise in CO_2 tension in a blood already diluted with an equal volume of Locke's fluid was certainly considerable. In a crossed-circulation experiment, in which arterial blood of a donor dog was run directly through the vascularly isolated carotids of the recipient animal, they got a reflex response in the recipient comparable with that in the donor; the rise in blood CO_2 amounted to 7 per cent (ambiguous as above), yet to this apparently marked change in blood CO_2

the donor is said to have shown only a feeble response. These workers refrained from committing themselves on the quantitative aspects involved, because of the lack of quantitative measurements of respiration, the paucity of data relative to blood CO_2 content, and the absence of information concerning pH and CO_2 tension. They state that on the whole the central response was more constant than the reflex, while the intensity of the reflex response was usually less (though occasionally greater) than that of the center after sinus denervation—a statement which strongly suggests that the response of the center was tested at the end of the experiment, in which case the condition of the animal after a prolonged bout of narcosis, perfusion, etc., must be taken into consideration.

The perfusion experiments of Bernthal (4) have already been mentioned and his results concerning the threshold of the carotid bodies to anoxemia commented upon. The reflex effects of increase in CO_2 tension of the blood were stated to have been weaker than those of anoxemia, to have shown more tendency than those of anoxemia to diminish during continued application of the stimulus, and the sensitivity to changes in CO_2 tension to have varied greatly in different animals; since no comparable statement appears in his comments on the sensitivity to anoxemia, it is probable that the latter was more consistent in his experiments, as it was in Schmidt's. Bernthal was the first to report data concerning the changes in CO_2 tension in the carotid blood required to elicit reflex effects: the smallest effective change was 15 mm. Hg, and the response to this was barely discernible. In the other examples listed, there was quite distinct (though by no means striking) reflex hyperpnea and a definite reflex vasoconstriction when the CO_2 tension was changed from 45 mm. to 76 mm., while a change from 45 mm. to 114 mm. produced strong reflex hyperpnea and vasoconstriction.

Simultaneously with Bernthal, Comroe and Schmidt (16) reported observations on the reflex and central effects of hypercapnia, made along with those on anoxemia, already mentioned in that connection. Their results agreed completely with those of Bernthal: the reflex effects of CO_2 (on respiration) tended to be distinctly less powerful, less consistent, and less well sustained than those of anoxemia. They found the smallest increase in CO_2 content by which a reflex hyperpnea was elicited to be about 4 vols. per cent. They also calculated the corresponding CO_2 tension to have been about 20 mm. Hg, but the pH estimations used in the calculation have subsequently been found faulty

and that figure is therefore worthless. The respiratory center was affected by increases in blood CO_2 smaller than 1 vol. per cent and minute volume of breathing was practically doubled when the change reached 2 vols. per cent. The reflex hyperpnea produced by a given change in CO_2 never approached the central in intensity, nor was it nearly as strong as the reflex hyperpnea produced in the same animal by anoxemia. These authors concluded that the carotid body receptors are distinctly less sensitive than the center to changes in CO_2 content of the blood, and believed their results to indicate that the normal regulation of breathing is accomplished without changes in chemoreceptor activity.

This conclusion has been contested by Heymans and Bouckaert (45), who point out—quite justly—that the reflex hyperpnea is necessarily diminished in intensity by loss of CO_2 through the expired air, thus reducing the chemical stimulus to the center, while during inhalation of CO_2 that is not the case. Their argument does not, of course, apply to the demonstrated differences in threshold to CO_2, but only to the differences in intensity of the reflex and central hyperpneas. Nor does it take account of the fact that, in the experiments of Comroe and Schmidt, anoxemia was proved to be capable of producing a much more intense reflex hyperpnea than hypercarbia, thus indicating that the preparations were capable of showing a strong reflex hyperpnea even though it meant reduction in the CO_2 tension of the blood.

Heymans and Bouckaert (45), however, state that the carotid chemoreceptors have a lower threshold than the center to CO_2, that they react more rapidly than the cells of the center, and that they preside over the rapid and fine regulation of breathing by CO_2. In support of this, they cite the results of an experiment on a dog in which the external carotid and occipital arteries were ligated on both sides, leaving the internal carotids open; the carotid innervation was left intact on one side, severed on the other; the vertebral arteries were also ligated. When "an appropriate quantity" of sodium bicarbonate solution of 2 to 3 per cent strength, buffered with CO_2 to pH 7.3, was injected into the innervated carotid, there was an immediate hyperpnea, whereas on the denervated side 10 to 20 times as much had to be injected before the respiratory center was stimulated, and then the effect was slower in onset and weaker, though more progressive and prolonged than the reflex effect.

One objection to this experiment is that the animal may have been one such as we have occasionally encountered, in which chloralose, in ordinary dosage,

practically abolishes the response of the respiratory center to CO_2 while distinctly exaggerating the sensitivity and the effectiveness of the carotid chemoreceptors. Another difficulty in drawing conclusions from such an experiment is pointed out by the authors themselves, namely, that the material injected must have been diluted with much more blood by the time it reached the center than had been the case when it reached the carotid body; they state that they do not believe that this dilution could amount to 10 to 20 times, though they give no reasons for that belief. Since the point at issue is not whether the carotid chemoreceptors can or cannot be made to respond to changes in CO_2 tension if the changes are sufficiently large, but whether the chemoreceptors are or are not more sensitive and rapid than the centers in responding to a given change, the validity of their conclusion depends on whether the stimulus acting on the center was comparable with that acting on the receptors. The CO_2 tension of a 2 per cent sodium bicarbonate solution, buffered with CO_2 to pH 7.3, would be 479 mm. Hg, that of a 3 per cent solution, similarly buffered, 724 mm., both at 38°C. In such an experiment this solution is injected into a relatively small amount of blood, since the carotid flow was intentionally restricted, and it would necessarily reach the carotid body almost instantly, with little opportunity for physico-chemical equilibrium to be established with the blood and none at all for dilution. The CO_2 tension of the fluid reaching the carotid body might therefore be enormous, and an immediate, powerful reflex response is not surprising. Between that point and the medulla, however, there is opportunity for a number of things to happen, all tending greatly to reduce the CO_2 tension of the blood. One is diffusion of CO_2 into the erythrocytes, to be converted into bicarbonate and bound as carbamate. Another is sweeping of a large proportion of the CO_2-rich blood forward, not only into the cerebral circulation but also, through the ophthalmic *retia mirabilia* (3), into the entire external carotid system, most of which is now being supplied with blood carried by the anterior spinals and internal carotids. In addition the CO_2-rich blood would be diluted with the presumably equal volume carried by the other internal carotid and with that brought by the anterior spinals, which may be considerable (witness the survival of one of Leonard Hill's dogs to become a household pet after ligation of both common carotid and vertebral arteries; Schmidt (64) gave data indicating that the anterior spinal-basilar communication may account for as much as 25 per cent of total cerebral blood-flow in dogs, for after occlusion of both carotid and both vertebral arteries outflow from the cerebral venous system was reduced on the average by 76 per cent). Doubtless there are other factors that would operate in the same direction. The situation in such an experiment seems to the reviewers to be too complicated and too imperfectly understood to permit any quantitative conclusions regarding the CO_2 tensions operating at the carotid body and at the respiratory center, and therefore regarding the respective thresholds and speeds of the responses of the two structures.

Recent experiments by Schmidt, Dumke, and Dripps (67) were intended to test Heymans' belief by the simplest methods available, i.e., by determining the elapsed time and the increase in arterial CO_2 tension required for the appearance of a distinct (50 cc.) increase in depth of

breathing, before and after inactivation of the carotid chemoreceptors, in lightly anesthetized vagotomized dogs subjected to slowly rising CO_2 tension in the blood. In the 6 best-conducted experiments the average elapsed time was 1 minute 15 seconds with the carotid innervation intact, 55 seconds with it inactivated—i.e., a tendency in the direction opposite to that to be expected if the carotid bodies respond more rapidly than the center. The average increase in arterial CO_2 tension at which the hyperpnea began was identical under the two conditions (3.3 and 3.4 mm. Hg), which gives no support to the belief that the chemoreceptors are significantly more sensitive than the center to increase in CO_2 tension in the blood.

Obviously, what is needed to decide the latter question is an adequate amount of valid quantitative data regarding the sensitivity of the center and the sensitivity of the chemoreceptors to changes in the CO_2 tension of their environment, under comparable conditions. Concerning the threshold of the center, the experiments just cited are probably valid for the vagotomized dog, lightly anesthetized with morphine and chloralose, and breathing 1 to 2 per cent CO_2 in oxygen: the average increase in arterial CO_2 tension at which depth of breathing was increased by 50 cc. (or more in some cases), with the carotid bodies inactivated, was 3.4 mm. Hg. This is probably higher than the actual threshold of the center because, to be certain that hyperpnea was actually beginning, it was necessary to wait for a second deeper breath, and by the time the blood was collected a third breath often had been taken; frequently these succeeding breaths were deeper than the first one. The actual threshold of the center to CO_2 was therefore probably less than 3 mm. Hg, but that figure may be taken as a conservative estimate of the order of sensitivity involved in such experiments.

The sensitivity of the chemoreceptors to changes in CO_2 tension (and pH) has been under investigation in this laboratory for more than a year. The animals (dogs) are lightly anesthetized with morphine and chloralose, vagotomized, and both carotid bodies are perfused with oxygenated Locke's solution, this being preferred to blood because with it one variable can be dealt with at a time. Results so far obtained (66) justify the statement that the greatest sensitivity to be expected corresponds with a change of about 10 mm. in CO_2 tension, for this is encountered only in exceptionally sensitive preparations. (In one case, as already mentioned, a 5 mm. increase coincided with slight though distinct reflex respiratory stimulation, but this could not be repeated and has never been duplicated.) In the majority of animals a change

of 15 to 20 mm. was required. Since these were perfusion experiments with saline fluid, the propriety of applying the results to the intact animal may be questioned. As far as we have been able to determine, saline perfusion *per se* does not diminish, and often definitely enhances, the activity of the carotid bodies, provided that the solutions are prepared with precautions similar to those required for successful perfusion of the mammalian heart. In any case, no other data are available at present.

The two thresholds to CO_2 are therefore about 3 mm. for the center, about 10 mm. for the carotid chemoreceptors, in the vagotomized, lightly anesthetized dog. Whether this ratio is or is not applicable to the intact unanesthetized animal or man is of course unknown; all information now available inclines the reviewers to believe that the abnormalities which were inevitable in the above-described experiments decrease the sensitivity of the center more than that of the carotid bodies, so that the ratio in the intact animal should be even more favorable to the center—certainly no less so.

With regard to the relative *strengths* of the reflex and central responses to CO_2, the burden of evidence leaves little doubt that the central is by far the stronger. The mere fact that the response of the whole animal to inhalation of CO_2 is not significantly altered by inactivation of the chemoreceptors seems to be irrefutable evidence that the latter do not play prominent or essential parts in the hyperpnea of CO_2. Heymans, on the contrary, still holds (45) that the reflex effect is stronger than the central because a reflex hyperpnea can be maintained (in perfusion experiments) by increased CO_2 tension in the carotids even though the CO_2 tension acting on the center is reduced by the concomitant hyperpnea. If the reflex factor really is stronger than the central, the reflex hyperpnea should produce a fall in arterial CO_2 tension greater than the increase in CO_2 tension required to set up the reflex. This is almost certainly not the case. In the reviewers' experience the reflex hyperpneas produced by increases in CO_2 tension smaller than 20 mm. have been relatively slight and not well sustained; only when the increase exceeded 20 mm. did the reflex hyperpnea become strong and persistent during the exposure. Bernthal's (4) experience apparently was similar. A fall in arterial CO_2 tension of the order of 20 mm. is not likely to be demonstrable as a result of the relatively slight reflex hyperpnea due to an increase of less than 20 mm. in the CO_2 tension of the fluid in the carotids. Again, if the reflex effect of CO_2 is greater than the central it should have been possible for those who have done

crossed-circulation experiments to show that inhalation of CO_2 by the donor animal produces a greater hyperpnea in the recipient than inhalation of the same mixture by the recipient under comparable circumstances, but while this has been repeatedly shown to be true of anoxemia, it has never been shown for hypercarbia. It is true that Gayet, Bennati, and Quivy (31) cite an experiment in which the donor dog's response to a 7 per cent rise in the CO_2 content of its arterial blood was smaller than the recipient's reflex response, but a result such as this can only mean that, because of deeper narcosis or other factors, the donor was less reactive than the recipient; the real test would then be to compare the recipient's own response to a given change in its blood with that elicited reflexly. This was done by Comroe and Schmidt (16), with results that have already been described (p. 129).

3. Hydrion concentration. This aspect of the problem has received little quantitative study. Heymans, Bouckaert, and Dautrebande (46) showed, by perfusion experiments, that reflex hyperpnea could be produced by changes in pH of the fluid, but they pointed out that the requisite changes were beyond the range pH 7.1 to 7.6. Since then a number of workers (see 47, 36) have demonstrated respiratory stimulation on intracarotid injection of acid, depression on similar injection of alkali, and the reflex nature of these responses was proved by their occurrence only if the carotid innervation was intact. Such results are of course purely qualitative; they cast no additional light on the relative sensitivity of the chemoreceptors and of the center to pH changes. Comroe and Schmidt (16) found no evidence of sensitivity of the carotid chemoreceptors to changes in pH in the blood, for blood to which HCl had been added was no more stimulant than the unacidified residue of the sample; their estimations of pH are now known to have been faulty and these particular results are valueless. Recent experiments (66) have given very different results, for distinct reflex respiratory effects have been produced quite regularly by changes of as little as pH 0.1 (phosphate buffer solutions, saturated with oxygen and free of CO_2). With larger changes (pH 0.4–0.6) intense reflex hyperpnea has been frequently observed. These recent studies show that pH changes are capable of stimulating the chemoreceptors almost as strongly as anoxia, and much more strongly and consistently than increased CO_2 tension, unless the latter is very great.

The threshold of the carotid chemoreceptors to changes in pH (accepting the lowest figure for which there is any experimental basis) therefore appears to be at about pH 0.1 in the dog under the conditions specified in the discussion of the CO_2 threshold.

An attempt to summarize graphically the relative sensitivities and strengths of the reflex and central components of the total (respiratory) response to anoxemia, hypercarbia, increased acidity, and cyanide (or lobeline) appears in figure 1. The presentation pertains to the anesthetized dog. It is schematic, but the relative thresholds, and the relative intensities associated with increasing strengths of the stimuli, are in accordance with the available experimental data. These, in the case of the anoxemia and hypercarbia graphs, are derived from the work of Comroe and Schmidt (16), supplemented by data obtained in recent experiments (66); the pH data are based entirely on the recent studies;

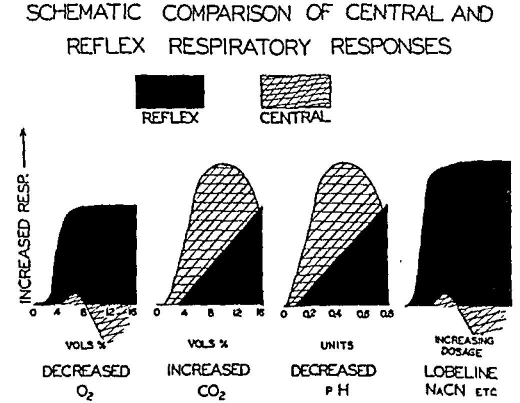

Fig. 1

the graph for cyanide (or lobeline) represents impressions rather than actual measurements. Since figures for changes in gas tensions in experiments like this are very scanty, we have used percentages of both oxygen and CO_2 in the blood; the relationships would certainly not be modified significantly if gas pressures were used instead.

The important points which these graphs are intended to bring out are as follows: the hyperpneas of anoxemia, cyanide, and lobeline are to an overwhelming degree reflex in origin; a component due to direct stimulation of the center is slight, has a relatively high threshold, and the margin of safety between central stimulation and depression is a

narrow one, whereas no depression of the reflex receptors by these agents is shown since none has yet been demonstrated. The hyperpneas of increased CO_2 and acid, on the other hand, are shown to begin as purely central affairs; the reflex factor comes into play only when the central response is nearing its peak, but the reflex stimulation becomes more and more important as the central response changes from stimulation to depression, as it would at high CO_2 percentages or hydrion concentrations. The maximum reflex effect of CO_2 or acid excess can equal that of anoxemia if the excess is great enough, while that of cyanide or lobeline can exceed the others (see p. 124). The maximum total hyperpnea produced by CO_2 or acid is greater than that of anoxemia, approximately equal to that of cyanide or lobeline. The shapes of the anoxemia and CO_2 graphs indicate (as was actually found by Comroe and Schmidt) that the reflex hyperpnea increases rapidly with decreasing oxygen percentage in the blood and soon reaches a maximum, while in the case of CO_2 the reflex factor increases progressively with the rising CO_2 content of the blood. The resemblance of these relationships to those illustrated by the dissociation curves of oxygen and carbon dioxide in the blood was pointed out by Irving (52).

III. THE PART PLAYED BY CHEMORECEPTOR ACTIVITY IN THE CONTROL OF RESPIRATION. On this question two distinct viewpoints have been developed. According to one, the chemoreflex system plays a significant part in the control of breathing under all circumstances; this view, sponsored by Heymans and his co-workers, has been adopted by most of the subsequent workers in the field (Wright, Gayet, Euler, Stèlla, Bernthal, Winder, Gesell, etc.). The other holds that the reflex system is too insensitive to respond to the slight changes in CO_2 tension or pH of the blood associated with respiratory control under ordinary conditions, but that by virtue of its great resistance to depressant agencies it comes into play as an accessory, supporting mechanism in emergencies such as anoxia and unusually great increases in CO_2 tension (Comroe and Schmidt; Schmidt). It now appears that the truth lies somewhere between these extremes.

The first of these viewpoints is not at all in harmony with available evidence concerning the thresholds of the chemoreceptors and the center. This becomes quite evident when the blood changes known to occur during the commonest and most normal respiratory adjustment, i.e., that to muscular exercise, are compared with known values for the thresholds of the chemoreceptors. Representative data are given in table 1. The threshold values in each case are the smallest for which

there is valid experimental support. It is assumed that the threshold of the whole organism to anoxemia is that of the chemoreceptors, and that—in the absence of information to the contrary—it is better to use our recent data bearing on chemoreceptor thresholds to CO_2 and pH, even though they were obtained in dogs, than to resort to inferences unsupported by actual evidence.

These data show clearly that in this individual the increased pulmonary ventilation during exercise must have been due to the increased acidity of the arterial blood, for the arterial CO_2 tension actually decreased slightly. The change in arterial pH that sufficed to produce this hyperpnea (a seven-fold increase in breathing is indicated by the

TABLE 1

Relation of changes in human blood during exercise to known thresholds of respiratory center and chemoreceptors

	Blood of A. V. B. (Journ. Biol. Chem., 73: 749, 1927)							Threshold of respiratory center of man	Threshold of chemoreceptors
	At rest (O_2 uptake = 250 cc./min.)			Exercise (O_2 uptake = 1750 cc./min.)					
	Artery	Vein	A-V difference	Artery	Vein	A-V difference	Changes in arterial blood		
O_2 tension (mm.).....	78	40	38	75	31	44	-3_1	More than -23*	-23 (Ellis)
CO_2 tension (mm.)...	40	45.4	5.4	38	54.8	16.8	-2	Less than $+1.5$*	$+10$ (dog)
pH (serum)........	7.425	7.399	0.026	7.351	7.278	0.073	-0.074	-0.0006*	-0.1 (dog)

* These figures are derived as follows: for anoxia, the central threshold is taken to be higher than that of the chemoreceptors; for CO_2, a 1.5 mm. increase in alveolar (= arterial) CO_2 tension suffices to double the resting ventilation (Haldane, 41) and the threshold must be lower than this; for pH, an increase of 1.5 mm. in CO_2 tension means a fall of 0.012 in pH, and one-twentieth of this is enough to affect breathing (Haldane, 41, p. 185).

change in oxygen uptake) was still well below any demonstrated sensitivity of the chemoreceptors to pH changes. The 3 mm. change in arterial oxygen tension was too small to have any effect on the chemoreceptors. As far as existing information goes, a response such as this must have been due entirely to a direct effect on the center. The size of the gap between the sensitivities of center and chemoreceptor is indicated by the figures for arterial and venous blood: if the latter were suddenly to replace the former in the arteries, the changes would still be insufficient to reach the threshold of the chemoreceptors (except in regard to oxygen tension) during the resting state, and during exercise the rise in arterial CO_2 tension thus produced would be of an order that

suffices to elicit a rather slight reflex hyperpnea in favorable experiments on dogs.

The weakness of this argument is naturally the comparison of data on chemoreceptor thresholds, obtained by perfusing saline fluids through the carotid bodies of anesthetized dogs, with data obtained from a study of the blood of unanesthetized man. There are no data on chemoreceptor thresholds to CO_2 or pH in man, and there is no prospect of obtaining them by direct experimentation. It is undeniable that the sensitivity of the reflex system in intact man may be greater than it was in the dogs, but we think it very improbable that the gap between the two sets of values would be closed by such data, first, because the chemoreceptor thresholds to anoxemia, as directly determined in animals, were not very different from the threshold of intact man to anoxemia (p. 122), and second, because the sensitivity of the chemoreflex system of decerebrate (but unanesthetized) dogs to cyanide is consistently increased by the anesthetic (chloralose) used in the dog experiments although the reactivity of the center (to CO_2) is unchanged or reduced (68). This takes no account of the artificialities of the perfusion; we have no data for evaluating that factor. The comparison shown in table 1 is advanced as simply representative of the data that are available at present and in hopes of crystallizing opinion on this subject.

The second viewpoint implies that the chemoreceptors are not active under normal conditions and become so only in unusual (emergency) circumstances typified by anoxemia, the marked hypercarbia associated with severe respiratory depression, and the action of certain drugs and poisons. This has become untenable in view of the continually increasing burden of experimental evidence indicating that the reflexes are active under normal conditions, and although the evidence is probably not as overwhelming as it seems there is little doubt that this view should be modified. The evidence, as usual, consists both of the simple denervation experiment and the more complicated perfusion procedure; studies of action potentials in the sinus nerve have also been used to elucidate this problem.

Among those who have done the *simple denervation experiment*, Selladurai and Wright (71) and Euler and Liljestrand (26) found (in cats) that the volume of breathing tended to be less after carotid denervation than before, and Euler (25) has recently presented additional evidence in the same direction. Euler and Liljestrand also found that the percentage of CO_2 in the alveolar air rose distinctly after the dener-

vation. All concluded that reflexes from the chemoreceptors are continuously effective even during quiet breathing. Witt, Katz, and Kohn (83) reached the same conclusion from experiments on dogs, in many of which respiratory failure occurred after bilateral (or even unilateral) denervation of the carotid reflex zone.

The propriety of drawing the above general conclusion from such experiments may be questioned on several grounds. One is the influence of the narcotic: there is no doubt that when the center is heavily drugged reflexes from the chemoreceptors may remain effective although the response of the center to CO_2 inhalation is reduced or lost. The reflexes then become more and more important (56, 16, 65), and the results of denervation under such circumstances may have no relation to more normal conditions. This is particularly applicable to the experiments of Witt, Katz and Kohn, but it also applies to the others. Selladurai and Wright found that decerebrate cats showed less consistent effects of this sort than narcotized animals. Another objection is trauma: in most cases the denervation was accomplished by mass ligations, and Witt, Katz and Kohn also used a phenol solution, absorption of which might have been a factor in their results. In any event, the effect of a similar dissection without sinus denervation does not seem to have been tested. Stella (75) dissected out the sinus nerves at the outset of the experiment and found clean section of them to elicit (in dogs) no consistent effect on the minute volume of breathing: depth tended to decrease, rate to increase. Still another factor is the rise in blood-pressure following the denervation, which may be very marked in reactive animals; this could of itself be responsible for changes in breathing, but the possibility does not seem to have been taken into consideration.

Observations bearing on this point were made recently by Schmidt, Dumke and Dripps (67) on lightly anesthetized, vagotomized dogs whose carotid pressoreceptors were divided while leaving the chemoreceptors functioning. When—during oxygen inhalation—the sinus nerves were blocked (with procaine)—which could be done without further manipulations and which now caused relatively little or no hypertension—breathing was not consistently affected and there were no significant changes in the pH, CO_2 content or CO_2 tension of the arterial blood. Precautions were taken to demonstrate the activity of the chemoreceptors (by intracarotid injections of cyanide) and of the center (by inhalation of CO_2). These experiments cast serious doubts upon the importance of the tonic activity of the carotid chemoreceptors

maintained by the CO_2 tension or pH present in arterial blood during quiet breathing without anoxemia.

The fact that breathing may be depressed (sometimes markedly) by inactivation of the sinus nerves in anesthetized dogs has been confirmed by Gesell and Lapides (37) and by Schmidt, Comroe, and Dripps (66), under conditions such that trauma and irritation were obviated (block of the exposed nerves by cold or procaine). Whether this justifies the conclusion that the chemoreceptors are active under conditions normally associated with quiet breathing depends, of course, on the experimental conditions: if the animal was deeply narcotized and its breathing correspondingly depressed, such a result only furnishes further proof that the chemoreceptors are active under those circumstances.

Perfusion experiments bearing on this aspect of the subject were made by Bernthal (4), who found that the vasomotor activity of the chemoreceptors was diminished when the oxygen content of the perfusing blood was raised above normal values, when its CO_2 tension was reduced, and when its acidity was diminished by the addition of Na_2CO_3. He concluded tentatively that—"the carotid body region is the source of a tonic chemo-reflex vasoconstrictor influence and that this is dependent upon the carbon dioxide and oxygen tensions of arterial blood." Even though the indicator of change in chemoreceptor activity which Bernthal used was probably an unusually sensitive one (see p. 144), the above conclusion remains unaltered in principle; the importance of the tonic influence may however have been exaggerated.

Additional perfusion experiments were made by Bernthal and Weeks (5) who pumped shed dogs' blood through the carotid bodies of dogs while maintaining constant the activity of the pressoreceptor system by means of a separate perfusion system. They found that cooling of the blood perfusing the carotid bodies caused reflex depression of the respiratory and vasomotor centers, while warming the blood had the reverse effect. They attribute these results to alternate inactivation and stimulation of the chemoreceptors in the carotid bodies. They were mainly interested in the nature of the regulation of chemoreceptor activity (see p. 149) and probably for that reason neglected to give data relative to the chemical state of the perfusing blood. In the absence of such information it is impossible to decide concerning the applicability of these results to the question of tonic chemoreceptor activity maintained by the CO_2 tension normally encountered in the arterial blood.

These experiments were recently repeated by Schmidt, Comroe, and Dripps (66), with the difference that a saline fluid was used instead of

blood, to obviate the complex changes that occur in blood when its temperature is varied (see p. 151). They fully confirmed the results of Bernthal and Weeks: cooling of the perfusing fluid caused reflex respiratory depression or apnea and warming caused hyperpnea. But they added the further observation that a result of the same sort could be obtained when the perfusing fluid was saturated with oxygen, free of CO_2, and of pH 7.5, i.e., when there was no known stimulant there to keep the carotid bodies active. (The oxygenation was proved adequate by the occurrence of apnea when such a fluid was substituted for one made anoxic by saturation with nitrogen or nitrous oxide.) These results indicate either that some of the chemoreceptors have so great a sensitivity to pH, CO_2 or anoxia that they are active when all of these factors are present in subnormal quantity (greatly subnormal in the case of CO_2), or else that there are receptors here that respond to something other than these three known excitants and are not strictly chemoreceptors at all. The possibility of temperature receptors was suggested by those authors, but they have adduced no evidence in support of that idea. It is equally possible that the receptors in question are continually activated by pH (see p. 153). In the recent experiments of Schmidt, Dumke and Dripps (67) there was no consistent depression of breathing when the chemoreceptors were inactivated during oxygen inhalation, even though the arterial plasma was distinctly more acid than pH 7.5 (the average was pH 7.4); this suggests that the amount of tonic activity maintained in ordinary chemoreceptors by pH 7.5 is negligible. But the same statement can also be made about tonic discharge from receptors activated by temperature, for this must also have been excluded when the sinus nerves were blocked, and if breathing was not distinctly depressed by this procedure it could not have been affected to an important degree by any sort of impulses from the carotid bodies during quiet breathing of oxygen. At present it seems most likely that striking results from the cooling experiment represent an unusual state of affairs rather than the usual one, and that animals showing such responses would be included among the small group that show distinct respiratory depression when the chemoreceptors are inactivated under resting conditions.

Studies of the *electrical activity in the sinus nerve* in relation to chemical excitation were first reported by Heymans and Rijlant (50) (in rabbits), but the published record is extremely difficult to evaluate. Subsequently Bogue and Stella (7), Samaan and Stella (63), Zottermann (88), and recently Euler, Liljestrand and Zottermann (29) have in-

vestigated this problem. All have used cats, and all have agreed that, in the sinus nerve of this animal, it is possible to record action potentials whose characteristics are different from those of the pressoreceptors: the former bear no relation to heart beats, are smaller, persist when the carotid pressure is lowered nearly to zero, increase progressively during asphyxia or hypercarbia, and continue for a long period (up to 30 minutes—Bogue and Stella) after cessation of the heart beat. Stella and co-workers, and Zottermann, both attempted to eliminate the fibers from the pressoreceptors by careful dissection, and both reported almost (though not quite) complete success in removing pressoreceptor activity of the sort studied by Bronk and Stella (13), while retaining this other type of electrical discharge. Zottermann believed that the latter comes from fibers smaller than those concerned in the pressure reflexes. Samaan and Stella reported that this discharge diminishes or ceases when the arterial CO_2 tension falls below 33 to 35 mm. Hg; in the example given, there is a small discharge at 35 mm. tension, more at 50, again more at 59, and still more at 72. They conclude that a tonic excitation of the respiratory center is thus proved to be set up in the carotid chemoreceptors by the CO_2 tension normally present in the arterial blood. Euler, Liljestrand, and Zottermann (29) have kindly placed at our disposal a copy of the proof of their paper, which is now in press. They confirm Stella's conclusions in the main, though reporting that some chemoreceptors are active at CO_2 tensions below 30 mm.; they also find greater sensitivity to anoxia than has been reported hitherto, for there were signs of reflex activity as soon as the arterial oxygen saturation fell below 96 per cent, which was considerably higher than the 89 to 92 per cent oxygen saturation that they found in the arterial blood of their cats when spontaneously breathing air. From these results they conclude that some tonic chemoreceptor activity is maintained, even under resting conditions, by the oxygen as well as the carbon dioxide tensions normally present in arterial blood.

There is, of course, no doubt that the sinus nerve carries impulses from chemoreceptors and it is probable that these were responsible for the electrical activities studied in these experiments, but there is no actual evidence in any of the published reports to prove that to be the case. In the animal used (the cat) the carotid body lies in the bifurcation of the carotid, imbedded in the fibers from the carotid sinus, not anatomically separated as in the dog. The latter animal has been tried by several groups of workers, to the personal knowledge of the reviewers, and none have been able to duplicate the above observations although

the anatomical situation is more favorable for such an experiment than in the cat. While this probably means only that the impulses from the chemoreceptors are smaller in the dog than in the cat, it may at least be pointed out that in the cat there are quite direct communications between the sinus nerve system and the superior cervical sympathetic ganglion, and that nerve impulses in the (thoracic) sympathetic are known to be increased by asphyxia, decreased by overventilation and increase in blood flow (12). The possibility that electrical disturbances picked up in the sinus nerve may have originated in some part of the sympathetic nervous system appears not to have been considered, though it seems only reasonable that if such studies are to be used to elucidate chemoreceptor activity it is incumbent on those who so use them to start by proving beyond reasonable doubt that the activity studied arose from the chemoreceptors and could not have originated anywhere else.

A compromise viewpoint, which appears to be in accord with existing evidence on this subject, is as follows: Different chemoreceptors have widely different sensitivities to the stimuli furnished by the blood; a small proportion are so sensitive that they are continually active under any circumstances compatible with life, but the great majority only become active when the stimulus level is increased. As this occurs, those already active become more so and more and more new ones also begin to discharge impulses; a certain quantum of increased reflex activity is needed to elicit a measurable increase in effector response, and this corresponds with the measured thresholds. Once the stimulus level has risen to the point of involving the mass of receptors, a further increase in the stimulus produces a greater response than a similar increase had elicited previously because more receptors are now involved in each increment; in addition, if the stimulus was anoxemia, the shape of the dissociation curve of oxyhemoglobin shows that the drop in oxygen pressure becomes much sharper per unit diminution in the percentage of oxygen in the blood (fig. 1). It is probable that the number of receptors that are continually active under resting conditions is relatively small and their physiological importance slight. The important point is the amount of increase in the stimulus level by which enough increase in chemoreceptor activity is produced to elicit a distinct physiological response. This conception of the activity of the carotid chemoreflex system is in accord with the description of the "chemical potentials" (63, 29), with the above-cited evidence indicating tonic activity, and with available information on the subject of thresholds of

sensitivity. Whether different receptors respond to different stimuli or all to the same ones (see 77, 29) remains unknown. The same may be said about different receptors being involved in the respiratory and vasomotor reflexes (see 31, 25). The identity of the chemical stimulus will be considered later (p. 154).

IV. SPECIAL FEATURES OF CHEMORECEPTOR REFLEXES TO THE CIRCULATION. As pointed out above (p. 118) the vasomotor and cardio-inhibitory centers are affected in the same direction as the respiratory by changes in chemoreceptor activity. This was first shown by Heymans and his associates (see 47), and has been confirmed by a number of workers (see 32). The effective stimuli for the vasomotor reflexes from the carotid body have been shown to be the same as those concerned in the respiratory: anoxemia, CO_2 excess, and acid excess cause reflex hypertension, as do cyanide, lobeline, nicotine, sulfide, etc. (see 47). As for the sensitivity of the chemoreflex mechanism acting on the vasomotor center, the experiments of Bernthal (4) indicate it to be equal to that pertaining to respiration. This interpretation assumes that in his experiments the indicator of a response of the respiratory center (increased rate or depth of breathing) was as sensitive as that used to measure a response of the vasomotor center (decreased flow of blood through a fore-limb perfused at constant pressure); the propriety of this assumption may be questioned. However, these studies indicate that the sensitivities of the vasomotor and respiratory reflexes to the stimuli tested (anoxemia, hypercarbia, cyanide, carbonate, bicarbonate, lactic acid) are of the same order of magnitude. The strength of the stimulus required to produce a significant activation of the vasomotor reflex system in the intact animal, according to Gellhorn and his co-workers (see 32) is in the neighborhood of 7 per cent oxygen in the inspired air (without artificial respiration). Comparison with the 18 per cent oxygen percentage at which hyperpnea appears (see p. 121) suggests that to produce a physiologically important activation of the vasomotor reflex in the intact organism, a much greater stimulus is required than is true for the respiratory reflex. This assumes that direct comparison of experiments on anesthetized animals with those obtained on unanesthetized man is justifiable, which is open to serious question. Further information is desirable, but since the circulatory effects of anoxemia are less consistent than the respiratory, it may be difficult to obtain.

Concerning the significance to the organism of these vasomotor reflexes, the situation is quite analogous to that encountered in discussing the respiratory effects (p. 118). By denervation of the chemoreceptors

it has been shown that the direct effect of anoxia on the circulatory apparatus, like that on the respiratory, is a depressant one (71, 64, 25, 75, 11, 32, 15): acute anoxemia causes pure depression of circulation as well as respiration in the denervated animal, while the circulatory effects of CO_2 inhalation (like the respiratory) are not significantly altered (unless they are increased because of removal of the pressoreceptor influence). Gellhorn and Lambert (32) have demonstrated that the hypertensive effects of CO_2 and anoxia are synergistic, probably because the former stimulates the vasomotor center strongly (and the chemoreceptors weakly), while the latter stimulates the chemoreceptors strongly and depresses the center. As an additional factor, we suggest that the marked dilator effect which CO_2 has been shown to exert upon intracranial blood vessels may, by improving the circulation through the medullary tissues, increase the resistance of the vasomotor center to the disorganizing influence of reduction in the oxygen pressure in the blood; this would be especially important if reflex hyperpnea occurred, for without CO_2 in the inspired air the tension of that gas in the blood would then be lowered and the intracranial vessels would probably constrict because, as far as is known, the influence of CO_2 upon them is stronger than that of anoxia (69, 65).

Comroe (15) has recently suggested that part or all of the reflex described by MacDowall (55) may be due to impulses from the aortic bodies to the vasomotor center, and not, as was previously supposed, to impulses from pressoreceptors in the great veins. Apart from this feature—which implies that reflexes aroused in the aortic body by anoxia (or other stimulant in unusual strength) maintain the activity of the vasomotor center in a manner comparable with the support of the respiratory center provided by carotid body reflexes when the latter center is profoundly depressed (56, 16)—there is no evidence that these reflexes are essential to the normal control of blood pressure though they are responsible for all of the hypertension of anoxia. As Gellhorn and Lambert (32) point out, the circulation is influenced primarily and most powerfully by impulses from the pressoreceptors, while the respiratory center is influenced predominantly by impulses from the chemoreceptors. The bradycardia aroused in the carotid bodies by chemical excitants appears to be elicited only by stronger stimuli than those which suffice for a minimal respiratory response, but this aspect of the subject has not been investigated systematically.

V. Partition of the total effect between the carotid and aortic receptors. This question can properly be considered in conjunction with the circulatory effects because it is in that connection that attention has been attracted

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V. PARTITION OF THE TOTAL EFFECT BETWEEN THE CAROTID AND AORTIC RECEPTORS. This question can properly be considered in conjunction with the circulatory effects because it is in that connection that attention has been attracted

to it. The original experiments of Heymans and Heymans (49) were such as to preclude any study of vasomotor reflexes from the aortic region because they were carried out on animals whose spinal cords were transected in the cervical region. Consequently the possibility of vasomotor reflexes of the same nature as the observed respiratory effects did not arise. The carotid reflex system proved so amenable to experimentation that the aortic region was neglected by all (except the anatomists) until the recent studies of Comroe (15). He found that, although there were wide individual variations, in dogs there was a definite tendency for the carotid chemoreceptors to produce the larger part of the total hyperpnea of anoxemia, cyanide or lobeline, and for the aortic receptors to account for most of the concomitant hypertension. In cats the individual variations were even wider than in dogs, but the carotid receptors of the average cat tended to be more important to the vasomotor response than was the case in the average dog. Additional reasons for believing that the aortic chemoreceptors are the major factor in the vasomotor response of the dog to anoxia were, first, that denervation of the carotids did not diminish, but actually increased the total effect, and second, that injections of cyanide (or lobeline) into the aorta in the region of the artery supplying the aortic body caused an immediate, strong hypertension (and hyperpnea), while similar injections into the carotids usually caused no hypertension (though strong hyperpnea) as long as the vagi were intact.

Heymans and Bouckaert (45) and Gellhorn and Lambert (32) both point out that the above comparison is misleading because the stronger hyperpnea elicited from the carotid chemoreceptors must necessarily lower the arterial CO_2 tension more and thus diminish the chemical stimulus acting on the vasomotor center to a greater extent than is the case with the aortic reflexes. They support this belief by experiments which show that carotid body reflexes to the vasomotor center are distinctly enhanced by artificial respiration (curara was also used in Heymans' experiments). While this objection is undoubtedly valid if the point at issue is the intensity to which the carotid vasomotor reflex can attain, it does not modify the conclusion that, in the intact dog, the hypertension of anoxemia or cyanide is due much more to aortic than to carotid reflexes. In the great majority of Comroe's experiments hypertension was produced by anoxemia or cyanide before section or blocking of the vagodepressor nerves, while afterward the hypertension was greatly diminished or totally lacking although the hyperpnea was certainly no greater than before. He also found that the hypertension produced by intra-aortic injection of cyanide (or lobeline) tended to be greater when the respiratory response was very vigorous than when it was not, which indicates that the vasomotor stimulant effects from the aortic chemoreceptors are not abolished by the lowering of the CO_2 tension of the blood so produced. Thus it seems clear that, in the average dog, the vasomotor stimulant reflexes aroused in the carotid chemoreceptors by anoxia are too weak to produce a significant rise in blood-pressure in the face of the reduced CO_2 tension consequent on the reflex hyperpnea, the bradycardia arising in the carotid chemoreceptors, the cardioinhibitory and vasodilator reflexes brought into action by the carotid and aortic pressoreceptors, and the depression of the heart and vasomotor centers produced by systemic anoxia; artificial respiration and vagotomy remove enough of these opposing influences to permit the vasomotor stimulation to become evident. But since

no such favoritism need be shown to the aortic chemoreceptors to permit their capacities in this direction to be manifested, they evidently exert a much stronger influence on the vasomotor center in the average intact dog than the carotid reflexes do. The actual importance of this conclusion is probably not great because, in view of the individual and species variations encountered by Comroe, generalizations are unjustified.

Data concerning the *thresholds* of the aortic bodies are entirely lacking.

Brief mention may be made here of a number of observations, made in this laboratory (doubtless duplicated in others in which experiments are made along these lines), which indicate that the carotid bodies may sometimes give rise to extremely marked disturbances in the central nervous system, one of the outstanding manifestations of which is a great rise in blood pressure. One of these observations—made on a decerebrate cat—is already on record (64), but the others are not. The course of events in all of them is about as follows: during perfusion of both carotid bodies a strong stimulus is applied to them (anoxemia, lowered pH, cyanide, lobeline, or—in the decerebrate cat—asphyxia consequent on stopping the perfusion pump). The usual immediate hyperpnea begins, but instead of striking a fixed level or diminishing, it steadily becomes more intense. After a brief period (about a minute as a rule) generalized muscular activity begins, micturition and defecation may occur, and occasionally violent convulsive movements appear. During this phase breathing is very violent and there is a very marked hypertension. The animal sometimes does not quiet down after a disturbance like this until more of the anesthetic is given, and we suspect that it is a phenomenon of very light narcosis (borne out by its occurrence in the decerebrate cat). Perhaps it is related to the convulsions produced in unanesthetized animals by cyanide (see 56b).

The reason for mentioning these results at this time is to raise the question of interpretation of an unusually marked hypertension following exposure of the carotid chemoreceptors to a strong chemical excitant. The hypertension now under discussion is undoubtedly of vasomotor origin, but it may be argued that a response like this is of a character very different from the usual prompt chemoreceptor effect; one may be dealing here with an excitation strong enough to produce a generalized motor response in the central nervous system, in which the vasomotor center simply participates incidentally, as would be the case in the convulsions produced by strychnine or tetanus. Curarization would of course suppress all the muscular phenomena while leaving the vasomotor response. At present we have no explanation to offer for the occurrence of reactions like this, but they undoubtedly point to a far-reaching and powerful distribution of effects from the chemoreceptors, at least in some animals and under some circumstances. They also introduce a complicating factor in the interpretation of experimental results.

VI. Pharmacology. The first demonstration that chemoreceptor reflexes may be prominently concerned in drug actions was that by J. F. and C. Heymans (49), who showed that nicotine can stimulate the respiration of the dog by way of reflexes arising in the aorta. C. Heymans and his collaborators (see 47) subsequently showed that nicotine can produce even more striking effects in dogs through the carotid chemoreceptors, and added observations to indicate that lobeline, sodium sulfide, and potassium cyanide act similarly. All of these drugs

were shown to stimulate the chemoreceptors, whose sensitivity to them was much greater than that of the centers. The same conclusion was reached by Heymans et al. with regard to the action of nitrites, by Zunz and Tremonti (89) with regard to sparteine, and by Dautrebande (17) with respect to hordenine. These conclusions were confirmed with respect to cyanide in numerous experiments in Gesell's laboratory (see 36), and by Euler and Liljestrand (27), and for nicotine, lobeline, cyanide, and sparteine by Wright (85). A large number of other substances have since been reported to be capable of stimulating the chemoreceptors, such as trimethylamine (57), KCl (25, 80, 81), anabasine, coniine, and cytisine (2), acetaldehyde (42), and a long list of compounds chemically and pharmacologically related to choline. The latter deserve special attention.

The first observations of this sort were those of Dautrebande and Marechal (19), who found that carbaminoyl choline was a strong stimulant to the carotid chemoreceptors—an observation that was subsequently confirmed by Philippot (62) and Comroe and Schmidt (16). Acetyl choline was also found effective by Heymans, Bouckaert, Farber, and Hsu (48), and this has been confirmed by Philippot (62), Schweitzer and Wright (70), Winder (80), Euler (25), and Comroe and Schmidt (16). Philippot (62) tested a large number of derivatives of this sort, including choline and the above and other esters of that base, representatives of the groups of α, β, and γ methylcholine, and esters and ethers of β-ethyl, β-propyl, and β-butyl choline. His results indicated that ability to stimulate the chemoreceptors runs closely parallel to "nicotinic" properties, and was therefore strong in choline and its derivatives and weak or absent in the β-methyl choline series. This was substantiated by Comroe and Schmidt (16), who found additional support for the above generalization in the fact that the choline compounds were effective after atropine—an observation that was also made by Wright (70) and was confirmed by Winder (80). There are, however, some discordant results in the literature: DeWispelaere (82) reports stimulation of the receptors by the acetyl ester and ethyl ether of β-methyl choline, which Philippot (62) and Comroe and Schmidt (16) found ineffective, and Farber (30) obtained positive results with the carbamic ester of β-methyl choline, which Philippot found ineffective. It is possible that these discrepancies are due to differences in dosage, for in large amounts these substances can affect respiration indirectly through their circulatory actions, even after atropine (72). Other observations were absence of reflex stimulant effect from pilocarpine but presence from a "muscarine" of uncertain identity (80). Schweitzer and Wright (70) concluded that prostigmine stimulates breathing through this reflex system, but the phenomena described by them appear to be too complicated to warrant categorical statements. They point out that the respiratory effects of acetylcholine are triphasic: an immediate strong stimulant response (retained after atropine but apt to be lost after denervation of the chemoreceptors), a secondary depression or inhibition of breathing (coinciding with the fall in blood pressure and therefore lacking after atropine) and a delayed stimulant effect. The experience of the reviewers with dogs has been very similar, and it seems fair to conclude that the chemoreceptor component of the action of acetyl choline in the intact animal is limited to the immediate hyperpnea following intravascular injection of large doses; perhaps the chemoreceptors contribute to the abrupt bradycardia that is also produced.

In the present state of our knowledge it is impossible to fit these various drug

actions with a common denominator, although the coincidence of reflex stimulant capacity with "nicotinic" properties is suggestive as pointed out by Mercier et al. (57), Philippot (62), Anitschkov (2), and Comroe and Schmidt (16). The persistence of these reflex effects after atropine is in accord with the view that the action is "nicotinic." Euler, Liljestrand, and Zottermann (29) point out that a discharge in the sinus nerve is brought about by agents which also cause a discharge of postganglionic impulses from a sympathetic ganglion, viz. nicotine, lobeline, acetyl choline, and potassium. They found that the discharge in the sinus nerve produced by nicotine and lobeline was not diminished by intravenous injection of ammonia while that produced by anoxemia, hypercarbia, or cyanide was reduced or abolished, which leads them to suggest that the former agents act centrally to the point of action of the latter, possibly on ganglion cells intercalated in the pathway of the impulses from the chemoreceptors (see p. 143).

Many of these substances are stated to have been capable of stimulating the respiratory center directly, but for this larger doses (usually much larger) were needed than was the case with the reflex excitation. Potassium is perhaps an exception, for Euler (25) found only depression of the respiratory center by it; Winder (81), however, states that the respiratory depression so produced bears signs of a stimulant process. The nicotinic compounds are, of course, capable of producing their characteristic ganglionic effects apart from the chemoreceptors.

In view of the current interest in acetyl choline and potassium as intermediaries in physiological excitations, it is important to note that existing evidence gives little reason to ascribe an important rôle of that nature to them here. The doses of acetyl choline required to elicit definite carotid body excitation are of the order of 0.1 mgm. intravenously (Wright) or 2 to 5 μgm. by direct injection via external carotid (Euler) in the cat, 0.1 mgm. by intracarotid injection in the dog (Heymans et al.). Winder (80) states that 1 μgm. of acetyl choline caused a barely perceptible stimulation of breathing when injected into a carotid whose circulation was restricted to the lingual branch, but that dosage seems large for this agent under the circumstances; injected into a femoral artery of a dog, it would produce distinct vasodilatation in that leg, although it would certainly be diluted more than in this case. As for potassium, Euler's (25) results show that if the stimulant dose is exceeded, or is repeated frequently, depression or paralysis of the chemoreceptors occurs, as tested by the response to acetyl choline, and Winder (81) reports the same thing with respect to the cyanide response. Since (according to Euler and Winder) the very resistant pressoreceptors behave similarly in this respect, it seems probable that we are dealing here with a disorganization of function—a non-selective type of action—rather than a specific stimulation, though the distinction is, of course, an arbitrary one.

VII. THE EXPLANATION OF CHEMORECEPTOR ACTIVITY. Two different conceptions have been advanced in this connection. Comroe and Schmidt (16) pointed out that a suggestion made by Koch (54), namely, that the pressoreceptor fields are localized in regions which are adult representations of the branchial arch arterial system of the embryo, together with Boyd's (10) conclusion that the carotid and aortic bodies arise in large part from the mesoderm of the latter system, could be

made into a reasonable hypothesis to account for the location of these chemoreceptors, as well as for their resistance to adverse circumstances such as anoxia, acid-excess, and marked increases in CO_2 tension. Further development of this theme (Schmidt, 65) included the suggestion that the strong stimulant effects of anoxia on these structures, coupled with the relatively weak effects of changes in CO_2 tension, might represent the survival, in the air-breathing adult, of an organization of utmost significance to the water-breathing forebear (typified by the embryonic branchial artery system): a reflex system having the characteristics of the mammalian carotid body and located in the gills, where direct contact with the environment takes place, can be envisaged as being of utmost importance to the water-breathing animal, though not so to the air-breathing one. Marshall and Rosenfeld (56a) had previously come to the same conclusion in their analysis of the circumstances responsible for respiratory depression or failure from oxygen inhalation. Boyd (10) states that there is no evidence to indicate that a chemoreceptor system exists in the gills of fishes. However, it appears (verbal communications from L. Irving and A. Krogh) that the respiratory movements of fishes are more responsive to changes in oxygen tension of the surrounding medium than to changes in CO_2 tension. The correspondence of this with the known properties of mammalian chemoreceptors, and the contrast with those of the mammalian respiratory center, are suggestive. Comroe and Schmidt therefore postulated that "the reflex receptors seem to be specialized to respond to changes in the oxygen tension rather than the CO_2 tension of the blood, while the cells of the center are specialized to respond to the latter rather than the former."

Gesell and his collaborators have taken the very different viewpoint that the chemoreceptors are "parts of the chemosensory mechanism located outside the medulla" (Winder, 77), and have sought in various ways to obtain evidence that the conception developed by Gesell (35), to account for the chemical control of the respiratory center, by the acidity existing within its cells, is equally applicable to the chemoreceptors. This evidence is summarized in a recent review by Gesell (36) as follows:

"Conforming with the postulated rôle of metabolism, a local rise of temperature of the carotid body designed to augment the metabolic production of acid (carbonic and lactic) increases pulmonary ventilation and contricts the blood vessels (Bernthal and Weeks, 5). Conversely, a blockage of the nutrient artery designed to hamper the transport of

oxygen to and acid from the carotid body stimulates breathing and vasoconstriction (Winder, Bernthal, and Weeks, 78). Anoxemia and cyanidemia which are known to cause an accumulation of lactic acid produce the same effects through the chemoreceptors (Heymans et al, 47; numerous others are also cited). Direct experiments indicate that such an accumulation of acids resulting from glycolysis is an essential step in the production of anoxic hyperpnea for when anaerobic glycolysis is prevented by localized poisoning with mono-iodoacetic acid increased breathing is missing (from anoxia) though hyperpnea is still produced by localized hypercapnia (Winder, 77)."

Now it is very probable that changes in acidity *per se* play a large part in the regulation of chemoreceptor activity, but the evidence cited by Gesell in that connection is by no means compelling. The work of Bernthal and Weeks (5) has already been mentioned, but no attempt was made there to point out a serious objection to the conclusion drawn from it by Gesell. These workers apparently overlooked the marked shifts in the hydrion concentration of the blood of the dog which Stadie, Austin, and Robinson (74) demonstrated when the temperature of the blood was altered under conditions such that gas could not enter or leave it; a change in temperature from 40 to 20°C. produced a rise in serum pH amounting to nearly 0.4 (fig. 2 of the paper of Stadie et al.). Since the temperature range studied by Bernthal and Weeks was even wider than this (from 43° to 16° in the examples shown) there may have been shifts in pH of sufficient magnitude to explain all of the observed effects, for changes in pH of the order of 0.4 are sufficient to produce very strong reflex effects in reactive preparations (66).

The experiments of Winder, Bernthal, and Weeks (78) were made on dogs in which all vascular branches of the carotid system were tied except one external carotid; occlusion of this must then produce complete ischemia of the carotid body, and when that occurred hyperpnea gradually developed, to disappear instantly when blood was readmitted. Observations analogous to these were made by Schmidt (64), who, in some experiments, found that hyperpnea developed gradually when carotid perfusion pressure was reduced from a low level to zero; in one experiment on a decerebrate cat the stimulant response was so violent that generalized convulsions ensued, and they ceased immediately when the perfusion was reinstated. In view of what has subsequently transpired, reactions such as this must have been due to increased chemoreceptor activity (as was indeed pointed out by Samaan and Stella, 63). However, there is nothing in any of these experiments to indicate the

nature of the exciting agent. The inference is that it was a product of metabolism but there is no clue as to its identity in observations like these. One cannot even be sure that it originated in the chemoreceptors: it may have arisen in adjacent tissues, or it may even have entered the receptors from the venous side when the arterial pressure was lowered to zero. From the account of the experiments of Winder et al. it is not clear whether this was obviated: there was an occipital artery—axillary vein anastomosis in two of the three experiments cited, and in the third a vein running with the sinus nerve was intentionally left open. The point has some importance because if the chemoreceptors have a high enough metabolism to produce their own stimulant as quickly as this, one has difficulty in understanding their great resistance to depression by anoxia.

The cited evidence obtained from anoxemia and from cyanide injections has not revealed anything that bears directly on the mode of action of these agents on the chemoreceptors. Production of acid within nerve cells exposed to anoxia is assumed to occur; though Ingraham and Gellhorn (51) have recently reported that the pH of brain tissue is not lowered but raised by anoxemia, nothing has yet been learned about the chemoreceptors.

The experiments which had most direct bearing on this question are those of Winder (77), who found that mono-iodoacetic acid, perfused in proper concentration through the carotid bodies of dogs, in favorable experiments caused abolition of the response to anoxemia while that to hypercarbia was retained. This was taken to mean that since glycolysis and lactic acid formation are known to be suppressed by this poison in other tissues, the loss of the anoxemic response points to involvement of glycolysis and acid formation as "probably an actual link in setting up the local excitation by anoxia." Winder himself points out one reason why such a conclusion is scarcely justified, namely, the probable presence of considerable edema in the perfused carotid bodies as a result of the poisoning. The CO_2 used to equilibrate the blood for the test of hypercapnia was 35 per cent, and the corresponding tension in that blood (at 760 mm. atmospheric pressure) would be 266 mm. With that pressure behind it, diffusion of some of the gas might well occur through edema fluid into the chemoreceptors, and if they were not too severely poisoned they would respond, whereas the edema fluid would be expected to furnish a source of oxygen that might well suffice for the chemoreceptors for a considerable time in the face of perfusion with anoxic blood. These experiments may add to existing evidence indicating that CO_2 diffuses

more readily than oxygen in living systems, but they do not justify conclusions with regard to the intervention of local acid production in the excitation of chemoreceptors by anoxia.

The evidence presented by Gesell in support of this conception is therefore far from convincing. Recently Euler, Liljestrand and Zottermann (29) have reported results which support Gesell's belief, at least in part. Studying action potentials in the sinus nerve of the cat, they have found that the electrical changes elicited by anoxemia, hypercarbia, or cyanide are completely abolished by intravenous injections of ammonia, whereas those set up by lobeline or nicotine are not affected. They conclude that the former agents act by a common factor, most probably by increased acidity within the reacting cells, while the latter must have a different point of attack, which may be ganglion cells in the carotid body.

An additional reason for believing that the hydrogen ion may be a very important factor in regulating chemoreceptor activity is an observation made by Heymans, Bouckaert, and Dautrebande (46). They showed that when the fluid perfusing the carotid bodies was excessively alkaline changes in CO_2 content, markedly effective at more normal pH, were without any effect. They gave an example of a shift from 76 to 16 vols. per cent CO_2 in Ringer's fluid (at pH 8 and pH 7.9 respectively) without any result at all, whereas much smaller changes were effective at pH 7.4 (see p. 126). As far as we know, this observation has not been confirmed. We have attempted to do so with solutions buffered with phosphates (to permit control of pH independently of CO_2 tension) but were frustrated by precipitation of calcium when the solution was more alkaline than pH 7.6. In the experiments (66) in which the activity of the chemoreceptors was altered by changing the temperature of the perfusing fluid, the pH was the only known chemical factor that could have been responsible for the activity, and it is quite probable that some receptors are continually activated by the hydrion concentration normally present in arterial blood. The unexpectedly low threshold (pH 0.1) which our recent experiments have shown the chemoreceptors to possess to changes in this stimulus, together with its great effectiveness as a stimulant to them, have given us added respect for it as a factor in chemoreceptor control. It is desirable to know whether the receptors are consistently inactivated by diminution in the acidity of their environment. Further experiments along these lines are now in progress.

When it comes to choice between these two conceptions of chemo-

receptor activity, the reviewers wish to point out that since only a little is known about the identity of the activating and transport systems involved in the energy-yielding reactions within the nerve cell, and nothing at all about the corresponding systems in the chemoreceptor, it is quite impossible to decide whether the two are sufficiently similar to warrant the belief that biochemical processes demonstrated in one can be used to elucidate the other. It seems to us, however, that the chances of a negative answer to that question are considerably better than those of an affirmative, for the chemoreceptors are quite different from the nerve cell in embryologic derivation and functional characteristics. The fact that the receptors show only strong stimulation when exposed to anoxia of a grade that would depress or paralyze the nerve cell shows that the former either have a much lower metabolic requirement than the latter, or can obtain energy by routes that are not available to the nerve cell. In either event the intracellular oxidative systems are likely to be quite dissimilar in the two structures. On the other hand, as noted above, the importance of the hydrion factor now looms much larger than it did two years ago (65). At present we believe it best to hold simply that the activity of the receptors can be altered by a variety of chemical agents, including all of the usual products of tissue metabolism, and that the same end-result can probably be accomplished by intracellular phenomena of diverse nature. We now have no good reason to believe that all of the drugs which stimulate the chemoreceptors do so either by reducing oxidations within them, as was suggested by Schmidt (65), or by intracellular acid formation, as implied (though not stated) in the conception of Gesell.

VIII. MISCELLANEOUS. An internal secretion by the carotid body has been sought repeatedly, most recently by deBettencourt, Cardoso, and deVasconcellos (21), who cite the pertinent literature. None of these attempts has revealed the presence of a specific, potent agent of physiological importance.

Evidence that chemoreceptor reflexes may have a far-reaching influence throughout the central nervous axis was presented by Kaufman (53), who showed that stimulation of the carotid body by cyanide or sulfide at times reduced the response of the tibialis anticus to reflex electrical stimulation applied to the central end of the posterior tibial nerve; at other times increases were observed. Perhaps this is another manifestation of the relation alluded to above (p. 147) in connection with the occasional occurrence of convulsions as a result of strong carotid body excitation.

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PHYSIOLOGICAL REVIEWS

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EXPERIMENTAL HYPERTENSION

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From a historical viewpoint, it is of interest that approximately 100 years separated each of the following observations or discoveries: 1, the circulation of the blood by Harvey; 2, the direct measurement of the blood pressure by Hales; 3, the association of chronic renal disease and hypertrophy of the heart (hypertension assumed) by Bright, and 4, methods for producing a persistent elevation in the blood pressure of experimental animals. It was only 40 years ago that an entirely satisfactory method for determining the arterial blood pressure in man was devised. Just as a careful study of hypertension in man was delayed by the absence of a good method for determining the blood pressure, so have those interested in the problem from the experimental viewpoint been handicapped by lack of simple and accurate means of measuring the blood pressure in unanesthetized animals. The method of Hales, in which the height to which the blood would rise in a tube was determined following direct cannulation of the artery, was improved by employment of the mercury manometer (Poiseuille). The number of determinations which can be carried out in one animal by this method is quite limited. A number of investigators, including Allen (1), have determined the systolic and diastolic pressures in dogs by a slight modification of the auscultatory method as used in patients. This has been unsatisfactory in the hands of most observers, probably due to the uneven contour of the extremities of dogs. A special cuff for use in dogs has been described by Ferris and Hynes (2). The van Leersum (3) loop method in which the carotid artery is placed in a tube of skin is considered satisfactory by many but others have found that it gives very variable results. The Kolls-Cash (4) sphygomanometer allows one to determine both the systolic and diastolic pressures in dogs but it is difficult for one to master the technique. The same may be said for the manometric method of Hamilton, Brewer and Brotman (5)

which is particularly valuable for the measurement of the pressure in small unanesthetized animals. A plethysmographic method for determining the systolic blood pressure of rats was devised by Byrom and Wilson (6), (general anesthesia required), and by Williams, Harrison and Grollman (7), (warming of the animal but no anesthetic agent). This method may be suitable for use on other animals. Although it gives neither systolic nor diastolic levels but rather a figure which corresponds closely to the mean blood pressure, the direct puncture of an artery with a needle connected to a mercury manometer is probably the easiest and is one of the most reliable means of making repeated determinations of the blood pressure in unanesthetized trained dogs. Other methods, too many to enumerate, include the indirect ones of Griffith (8, 9) and of Grant and Rothschild (10). These are particularly adapted to use on small animals.

This review will deal only with experimental hypertension in which the evidence indicates that both the systolic and diastolic pressures are elevated. From the clinical side, it is known that a purely systolic elevation of blood pressure may be associated with aortic insufficiency, hyperthyroidism, arteriosclerosis of the aorta, heart block and arteriovenous fistulae. Some of these conditions can be produced in the experimental laboratory but do not represent true instances of hypertension. Consideration will not be given to the acute arterial hypertension that may be produced by agents such as epinephrine, paredrinol and other chemicals. Although the findings will not be given in detail, it should at least be mentioned that Appelrot (11) and subsequently Handovsky and Goormaghtigh (12, 13, 14) have found that the oral administration to dogs of large doses of "Vitamin D" (vigantol, calciferol) is accompanied by an elevation of blood pressure. Handovsky (14) quotes one experiment in which the pressure remained elevated for the 80 days that the substance was administered and the pressure then gradually declined to the control level. The mechanism responsible for the elevation of pressure has not been determined. Hypertrophy of the media of the arterioles, particularly of the kidney, have been found on histological examination. These alterations in dogs are modified by the removal of the thyroid or the parathyroid glands. Since changes in the arterioles of the kidney are rather marked, it is possible that the hypertension is due to renal ischemia. At any rate, this method is not among the better ones for producing chronic hypertension because the pressure declines if the medication is stopped and, thus far at least, it is not known to have any relationship to hypertension as seen in man.

Successful experimental investigations into the cause of hypertension have in general followed along two lines, one dealing with the elevation of blood pressure which may be produced by one of several procedures carried out on the central nervous system, the other dealing with the hypertension that is renal in origin. If undue space is allotted to the hypertension associated with renal ischemia, it is hoped that it will be excused on the basis of the great interest in and the large amount of work being done on this subject at the present time. For the recent revival of interest in this type of hypertension, Goldblatt is largely responsible. The types that are generally considered to be non-renal in origin will be considered first.

EXPERIMENTAL HYPERTENSION AND THE NERVOUS SYSTEM. I. *Carotid sinus denervation and aortic depressor nerve section.* Following the observations of Cyon and Ludwig on the depressor nerve and of Hering on the carotid sinus, it was shown by Koch and Mies (15) that hypertension can be produced experimentally by bilateral denervation of the carotid sinus and section of the aortic depressor nerve. These observations have been confirmed and elaborated upon by Heymans (16, 17) and by others. Interruption of the afferent nerve impulses which normally buffer or depress the activity of the circulatory centers in the medulla probably permits the vasoconstrictor and cardio-accelerator centers to exert greater effects on the blood pressure. Heymans (18) has observed arterial hypertension maintained at 250 to 300 mm. Hg for periods ranging from 9 to 26 months after section of the moderator nerves; and Nowak and Walker (19) have found that the elevation of pressure may persist for three years. Section of the cardio-aortic and carotid sinus nerves produced only a temporary rise in pressure in some of the dogs in which this procedure has been performed. Heymans (18) states that this is due to the presence of accessory fibers of the cardio-aortic nerves in the vagus or to the taking over of the functions of the moderator nerves by pulmonary and intestinal presso-sensitive nerves. The authors do not give in their publications the percentage of cases in which a prolonged elevation of pressure followed section of the moderator nerves. In a personal communication, Nowak states that at the outset he was successful in producing hypertension in only 30 to 50 per cent of dogs but that more recently hypertension has resulted in 90 per cent of cases. The operative procedure consists of excision of the carotid artery bifurcation (including the carotid sinus) and of the upper cervical portion of the aortic-depressor nerve. Using a slight modification of the procedure used by Heymans, it was found by Green, Degroat and McDonald

(20) that rabbits had only a transient elevation of blood pressure and that dogs had an unstable pressure with usually some elevation which tended to become stabilized subsequently at a level not greatly above the normal. The contradictory results are explained by Heymans as due to the difference in the operative procedure.

Heymans (18) found in dogs that removal of the paravertebral sympathetic ganglia and chain, from the stellate to the pelvic ganglion, prevents or causes the disappearance of this type of hypertension. On the other hand, Nowak and Walker (19) state that preliminary total sympathectomy fails to prevent a moderate rise in blood pressure when the moderator nerves are divided. Further, they found that bilateral splanchnicectomy causes only a temporary fall in existing hypertension produced in this manner and that sympathectomy has to be complete in order to abolish it. Heymans and Bouckaert (21) found that the blood of dogs rendered hypertensive by section of the moderator nerves possesses a higher degree of vasopressor activity than the blood of normal dogs. Braun and Samet (22, 23) state that denervation of the kidneys prevents the development of or abolishes the hypertension associated with section of the moderator nerves. Elaut (24) could not confirm these observations. The hypertension is unaffected by bilateral subtotal adrenalectomy and section of the splanchnic nerves (25).

Descriptions of the histological picture of kidneys of animals with this type of hypertension are contradictory. Nordmann (26) observed no lesions of the arteries or arterioles of the kidney and found only slight glomerular lesions. Goormaghtigh (27), on the contrary, found in hypertensive rabbits, hyperplastic and degenerative lesions of the renal arterioles in addition to lesions of the glomeruli and tubules. He states that the vascular lesions are secondary to the high blood pressure. Hoerner, Fontaine and Mandel (28) stated recently that there is no alteration in the function or histological picture of kidneys of dogs which had had hypertension for two years following section of the moderator nerves. The use of different animals may explain in part at least the contradictory results. Spontaneous arteriosclerotic lesions are not uncommon in rabbits.

All seem to be agreed that section of the moderator nerves results in an elevation of blood pressure but only Heymans and Nowak have obtained with fair consistency a persistent elevation. Insufficient figures are given to allow one to speak with certainty. One gains the impression that section of the moderator nerves usually results in a temporary rise of blood pressure and infrequently in a sustained elevation. The

results, although slightly contradictory, seem to indicate that the elevation of pressure does not result in histological alterations in the arterioles throughout the body. This type of hypertension differs from that due to renal ischemia in that there are greater fluctuations in blood pressure; the elevation is not as apt to be sustained and there are fewer pathological changes in the blood vessels.

The question arises as to whether this type of experimental hypertension induced by removal of the moderator nerves is related in any manner to hypertension as observed in patients. If there is a relationship, it is with essential hypertension rather than the nephropathic variety. It is known that increased vasoconstriction is present in essential hypertension and in experimental hypertension due to removal of the moderator nerves. However, it does not follow necessarily that essential hypertension is due to an abnormality of the carotid-sinus and aortic-depressor nerve mechanisms. As Nowak and Walker (19) have stated, the similarity lies in the end result rather than the cause. It is of interest that experimental hypertension due to removal of the moderator nerves and essential hypertension in man are usually affected very little by division of the splanchnic nerves.

II. *Increased intracranial pressure.* According to Cushing (29), an acute increase in intracranial pressure produces hypertension by causing cerebral anemia. Raab (30) has shown that central anemia stimulates the vasopressor centers and increases their sensitivity to carbon dioxide. Perfusion of the vasomotor center with acids causes a rise in blood pressure and perfusion with alkaline solutions causes a fall. Dixon and Heller (31) found that hypertension of months' or years' duration may be produced in dogs by the injection of kaolin into the cerebrospinal system, thereby causing an increase in intracranial pressure. In several of the animals the blood pressure fell rather abruptly after being elevated for three months. Griffith, Jeffers and Lindauer (32) produced hypertension in rats by the same method. In addition to an increase in the cerebrospinal fluid pressure (33), an elevation of both the systolic and the mean arterial pressure and of the capillary pressure were noted (34). Griffith and Roberts (34) found that an elevation of pressure usually did not occur before the fifth day following the injection of kaolin. In only the occasional rat did the hypertension persist for as long as two months, and the elevation was not permanent in any of them. Lindauer and Griffith (35) were unsuccessful in producing hypertension in cats by this method.

It has been found that this type of hypertension persists in both dogs

(36) and rats (34) following bilateral adrenalectomy if salt and a non-pressor extract of the adrenal cortex are administered. Braun and Samet (37) state that an elevation of pressure can be prevented or reduced to normal by renal denervation. They observed that denervation of one kidney resulted in a decline of the blood pressure, removal of the denervated kidney was followed by a return of the pressure to the hypertensive level. On the other hand, the blood pressure remained depressed if the normal rather than the denervated kidney were removed. Freeman and Jeffers (38) stated recently that sympathetic cardiac innervation, neural or humoral, is necessary for the full development of hypertension produced by increased intracranial pressure. Extensive sympathectomy extending from the fifth thoracic to the fifth lumbar did not prevent the elevation of blood pressure. Pick (39) found that the injection of a relatively small quantity of blood from a dog with hypertension due to kaolin into a normal dog or into a dog in which the kidneys had been denervated resulted in a marked elevation of pressure in the recipient for a number of hours. Injection of blood from a normal dog into a dog with kaolin hypertension had no influence on the blood pressure, but strangely enough, injection of blood from a dog with denervated kidneys into a dog with hypertension due to kaolin resulted in a decline of the blood pressure. Vogt (40) demonstrated, by perfusion experiments, pressor properties in the blood of animals with hypertension due to kaolin. No constricting effect was observed if an adrenalectomy had been performed on the donor of the blood.

The findings by autopsy of the dogs of Dixon and Heller (31) were essentially normal except for dilatation of the cerebral ventricles. The only abnormality noted in the kidneys was that the capsule was unusually adherent. One of these animals had had hypertension for 27 months. Hamperl and Heller (41) noted no gross or microscopic abnormalities in the aorta and in the arterioles of various organs of dogs which had had hypertension for eight months.

Despite the optimistic reports of some observers, one gets the impression that this type of hypertension cannot be produced with great regularity and that the elevation of pressure is usually not persistent. That the elevation of pressure is due to cerebral anemia seems to be fairly well established. Further histological studies of the arterioles of the occasional animal in which the hypertension persists for a year or longer should be performed.

III. *Cerebral anemia.* Nowak and Samaan (42) showed that acute anemia of the cerebral circulation alone causes a marked rise of the

general arterial blood pressure. They perfused the isolated head connected to the body only by the spinal vasomotor pathways. Blalock and Levy (43) state that a sustained elevation of the blood pressure does not usually follow the complete occlusion of the two common carotid and the two vertebral arteries. However, Nowak (19) has found that progressive occlusion of the various cerebral arteries may cause chronic hypertension. The vessels which are ligated include both the external and internal carotids, both vertebral arteries and the anterior spinal arteries. Due to the development of collateral circulation, these procedures do not always result in hypertension. Nowak (44) has recently modified his procedure to include ligation of both subclavian arteries distal to the origin of the common carotid and proximal to the origin of the vertebral artery. This hypertension whether of short or long duration is said to be maintained despite the buffering action of the carotid sinus and aortic depressor nerve mechanism.

The findings of Nowak have not been published in detail, only one blood pressure chart being given, and it would seem very doubtful if this method will prove to be a consistent one for producing hypertension. The elevation of pressure is due almost certainly to cerebral ischemia, and it seems likely that the pressure will decline in most instances as the collateral circulation to the brain increases. Therefore, the available evidence indicates that this is not a consistent method for producing chronic hypertension.

Experimental hypertension and the kidneys. There has been much speculation and controversy for many years as to whether or not diseases of the kidneys are a major factor in the pathogenesis of hypertension in man. There is clinical evidence that the kidneys are diseased in many patients with hypertension. Some pathologists have stated that diseased kidneys are found at autopsy in all patients who have had hypertension for a long time. Fishberg (45) lists the following clinical conditions in which disease of the kidneys or intrarenal blood vessels may lead to hypertension: 1, polycystic disease; 2, chronic pyelonephritis, sometimes unilateral; 3, renal amyloidosis; 4, mercury poisoning; 5, periarteritis nodosa; 6, other forms of renal arterial occlusion; 7, renal hypoplasia; 8, tumors of the kidney; 9, embolism of the renal artery, and finally and probably most important, 10, glomerulonephritis. It has been known for many years that obstruction to the flow of urine may result in hypertension. The greatest interest in recent years has been centered on attempts to find the etiology of essential hypertension which has been defined as a persistently elevated blood pressure of un-

known cause. The central problem has been that of finding the cause for the generalized vasoconstriction, and prior to the work of Goldblatt and associates (46), the earlier theories had been abandoned in the main and the kidney was not seriously considered as the most likely source of this effect. Vascular disease of the kidney itself was considered by most observers to be simply a part of a generalized disease process. Because of the experimental and possible clinical importance of the work of Goldblatt and of those who have used his method, the results will be considered in moderate detail. Other observations which may be of great importance include those of Masugi on the experimental production of glomerulonephritis.

Goldblatt (47) and Fishberg (45) have presented excellent summaries of the experimental evidence for the view that the kidney may play a major part in the development of hypertension. Their points of view will be reëmphasized and reference will be made to some of the more recent work.

I. *Experimental glomerulonephritis.* The early lesions in acute diffuse glomerulonephritis consist of a general swelling and proliferation of the endothelial cells of the glomerular capillaries together with an accumulation of inflammatory exudate within the loops. These changes result in ischemia of the glomeruli and in this one respect there is a similarity between acute glomerulonephritis in the human and hypertension in animals resulting from renal ischemia as produced by the Goldblatt (46) method. The prevailing impression is that the glomerular lesions of diffuse clinical glomerulonephritis are not due to direct invasion of the kidneys by microörganisms but rather to injury by the toxic products of organisms. Diffuse glomerulonephritis complicating various infections usually occurs not during the most active stage of the infection but during convalescence. This fact together with observations on serum sickness and other allergic phenomena suggested to Schick (48) and to von Pirquet (49) that the processes of immunization with the accompanying hypersensitiveness are in some manner connected with the development of glomerulonephritis following an infection.

The earlier literature dealing with attempts to produce glomerulonephritis was reviewed by MacNider (50) and by Leiter (51) in 1924 and by Longcope (52) in 1929. Leiter (51) stated, "Chronic glomerulonephritis has not been produced constantly or even frequently in an experimental animal. . . . Whatever changes were observed in the kidneys could not be interpreted as those of chronic glomerulonephritis."

Longcope (53) was the first to attempt to produce glomerulonephritis in sensitized animals. He produced renal lesions including glomerular changes by injecting repeatedly into various animals horse serum and egg-white. Duval and Hibbard (54) produced what was termed glomerulonephritis by the injection of the endotoxic principle of the streptococcus scarlatinae. The endotoxin was obtained from the viable scarlatinal cultures through the medium of the peritoneal cavity of the rabbit immunized against the homologous strain. Long and Finner (55) observed diffuse renal inflammation following the injection of tuberculin into the kidneys of swine made sensitive to this substance by the presence of mild tuberculosis. Bell and Clawson (56) produced a form of chronic diffuse glomerulonephritis in a monkey by repeated intravenous injections of streptococci over a period of four years. The blood pressure was not determined. Lukens and Longcope (57) produced both focal and diffuse glomerulitis in rabbits by the injection directly into the renal artery of heat killed hemolytic streptococci. Glomerulitis was observed much more frequently in rabbits in which an acute localized streptococcus infection had been produced previously by the intracutaneous injection of living hemolytic streptococci. Somewhat similar lesions were produced by McLeod and Finney (58) using suspensions of streptococcus viridans and by Blackman, Brown and Rake (59) employing an autolysate of type I pneumococcus. These and many other references which cannot be mentioned due to lack of space demonstrate the partial success that has followed the use of methods based on immunity reactions in attempts to produce glomerulonephritis.

It is rather generally agreed that the alterations in the kidneys produced by the methods referred to are not identical with the histological picture of glomerulonephritis as observed in patients. However, this aim has been approached more closely by Masugi (60, 61, 62), who produced what appears to be glomerulonephritis by the injection of anti-kidney serum. Anti-kidney serum was first used by Lindemann (63) in 1900 but it is to Masugi that credit is due for recent interest in this method. He found that by giving rabbits a series of parenteral injections of a suspension of rats' kidneys, the serum of the rabbits developed the property of producing glomerulonephritis when injected into rats. Similar results were obtained in a repetition of this work on rabbits in which ducks were used as the donor of the anti-serum. These findings have been confirmed by Arnott, Kellar and Matthew (64). Smadel (65) found that the anti-kidney sera contain a number of anti-

bodies capable of inducing a severe anaphylactoid reaction and also a nephrotoxic agent that affects the kidney primarily. The nephrotoxic effect is characterized by severe persistent albuminuria with casts and transient anasarca. Hematuria is observed when a severe anaphylactoid reaction is superimposed on the nephrotoxic injury. The nephrotoxic action of anti-kidney serum is removed by absorption with kidney cells or fat-free kidney tissue. Swift and Smadel (66) were able to prevent renal damage due to anti-rat-kidney serum by injecting previously a saline extract of perfused rat kidney. Smadel (67) found that the urinary abnormalities which developed after the injection of nephrotoxin continued until the animal died or was sacrificed. The early renal lesions changed into scarring of the glomeruli and tubules. Studies which were made 3 to 11 months after administration of nephrotoxin revealed chronic progressive glomerulonephritis and generalized vascular lesions. Moderate elevations of blood pressure after treatment with anti-kidney serum have been recorded by Masugi, by Arnott, Kellar and Matthew, by Smadel and Farr (68) and by others. Smadel and Farr state that an elevated blood pressure occurs only in those rats which develop a chronic progressive nephritis and that the hypertension may be influenced by dietary means.

It appears that Masugi and others who have used his method have produced a condition which resembles human glomerulonephritis very closely. However, it is not proven that the pathogenesis of the two is the same. The main point of interest lies in the demonstration that antibodies may be produced which damage the kidneys in a manner similar to that observed in human glomerulonephritis. This is particularly important in view of the belief that sensitization to bacterial infections plays a part in the causation of this disease in man.

II. *Bilateral nephrectomy.* As a preliminary to the following considerations, it should be emphasized that bilateral nephrectomy (69–76) does not result in an elevation of the blood pressure. It was shown by Cash (72) and confirmed by Blalock and Levy (76) that marked interference with the arterial supply to the kidneys results in a significant elevation of blood pressure, provided the entire arterial circulation, including that through the main artery, the smaller vessels in the pedicle, those in the ureter and the capsular vessels, is not completely occluded. In other words, complete absence of the kidneys does not cause a blood pressure elevation, whereas a marked reduction in the renal blood supply causes at least a temporary elevation of blood pressure.

III. *Obstruction to the flow of urine.* The first significant experiments

of this type were those performed by Rautenberg (77) in which temporary occlusion of one of the ureters of rabbits was produced. Following release of the occlusion and removal of the opposite kidney, a moderate elevation of blood pressure was observed. Hartwich (73, 74) and Harrison, Mason, Resnik and Rainey (75) found a moderate elevation of blood pressure following the complete occlusion of both ureters. It was found by Blalock and Levy (76) that the blood pressure returned to normal in approximately six hours following the removal of a single hydronephrotic kidney. Levy, Mason, Harrison and Blalock (78) found that early hydronephrosis is associated with a decrease in renal blood flow, little if any alteration in the arteriovenous oxygen difference, and a decrease in the oxygen consumption. The findings are similar to those encountered in renal ischemia (79) as produced by the Goldblatt method and it is likely that the mechanism of the production of the hypertension associated with each of these conditions is the same. The establishment of a uretero-venous anastomosis, thereby allowing the urine from a normal kidney to enter the venous system, does not result in hypertension (80). Denervation of the kidneys (81, 82) does not abolish the hypertension associated with hydronephrosis. Harrison and associates (83) found that the hydronephrotic kidney generally contains more pressor substances than its unobstructed mate. Williams, Wegria and Harrison (84) state that rats with spontaneous bilateral hydronephrosis have a marked elevation of blood pressure. Extracts of kidneys of rats with induced or spontaneous hydronephrosis cause a greater rise of blood pressure on injection into normal rats than do extracts of the kidneys of normal rats. Furthermore, the sensitivity of hydronephrotic rats to the renal pressor substance is somewhat greater than that of normal animals.

IV. *Subtotal nephrectomy by various methods.* The earlier work on partial nephrectomy includes that of Grawitz and Israel (85), Tuffier (86) and Bradford (87). The first evidence of hypertension associated with cardiac hypertrophy as a result of partial nephrectomy was presented in 1905 by Pässler and Heinecke (88). Many similar experiments have been performed since that time in which the amount of functioning renal tissue has been reduced by simple excision in multiple operations, by ligation of branches of renal arteries, or by nephrectomy combined with one of these procedures. Using one or more of these methods, arterial hypertension was noted in the chronic state by Janeway (89), Allen, Scharf and Lundin (71), Mark and Geisendorfer (90), Chanutin and Ferris (91), Chanutin and Barksdale (92), Wood and

Ethridge (93), Rytand and Dock (94) and others. A temporary elevation of blood pressure was found by Cash (95), Hartwich (73, 74), Ferris and Hynes (2) and others. Particularly important among these observations are those of Chanutin and associates (91, 92) who produced a marked and sustained elevation of blood pressure in rats by partial nephrectomy, the procedure consisting of polar ligation and excision of renal tissue. It was shown that rats which were deprived of approximately 80 per cent of their renal tissue developed progressive renal lesions, arterial hypertension and cardiac hypertrophy. Polyuria was a striking finding. They state the assumption seems warranted that in partially nephrectomized rats with renal insufficiency, an increase of the blood pressure is necessary to maintain an increased volume of urine in order to excrete metabolites in lower concentration. The particular importance of this method lies in the fact that it is the best one for producing hypertension in very small animals, with the possible exception of that of Drury (96), whereas the procedure of Goldblatt (46) is preferable in larger animals. A possible disadvantage for some types of studies is that the hypertension is probably associated with renal insufficiency. Pässler and Heinecke (88) and Chanutin and Ferris (91) noted a failure in producing hypertension in animals becoming cachectic shortly after or during the course of repeated operations.

Wood and Cash (97) observed an elevation of the diastolic as well as the systolic pressure in dogs following subtotal nephrectomy and state that the rise of pressure is not proportionate to the degree of renal insufficiency. Focal accumulations of fat within the media of smaller arteries and arterioles, frequently leading to a marked diminution in the size of the lumen, were found in rats following partial nephrectomy by Wood and Ethridge (93). It is their opinion that the progressive glomerular and arterial renal lesions may be interpreted as a natural sequence of hypertrophy and degeneration brought about by functional strain. Diaz and Levy (98) observed in hypertensive rats a decline of blood pressure following bilateral adrenalectomy. Destruction of the central nervous system was found by Dock and Rytand (99) to cause an abolition of this type of hypertension in rats. They conclude that if a pressor substance is present in the plasma of rats with renal hypertension, it has no direct vasoconstrictor effect but acts through the vasomotor center. They also found (100) that no significant decrease in blood flow per gram of active kidney tissue takes place as hypertension develops. Dock and Rytand (100) state, "Rats which become

hypertensive several months after subtotal nephrectomy do not have renal ischemia; the flow per gram of renal tissue is 19 per cent less than in rats a few days after subtotal nephrectomy, but the same as in rats with unilateral nephrectomy and without hypertension." These findings of Dock and Rytand which indicate an absence of a vasoconstrictor substance in the blood and a normal blood flow per gram of renal tissue are contrary to findings to be discussed later in hypertension as produced by the Goldblatt method.

V. *Kidney damage by various means.* The attempts to produce hypertension by the injection into the renal arteries of liquid paraffin (101), of insoluble Berlin blue (95), and of particles of charcoal (102) have been unsuccessful. Maegraith and McLean (103) reported the production of hypertension in rabbits by the injection of a suspension of Kieselguhr white into one renal artery. Further it was noted that renal denervation abolished the hypertension. Cressman and Blalock (104) have attempted without success to repeat these observations on dogs.

It was found by Dominquez (105) that the intoxication caused by uranium, radium, lead and vanadium does not result in a significant alteration of the blood pressure of rabbits. A moderate temporary elevation of blood pressure (106) which is abolished by renal denervation (107) was produced in rabbits by the repeated intravenous injections of sodium oxalate by Arnott and Kellar. These findings were not confirmed by Scarff and McGeorge (108).

Hartman, Bolliger and Doub (109) produced hypertension in dogs by the use of high voltage Roentgen rays. The alterations included extensive destruction of the renal parenchyma with replacement fibrosis and rather diffuse renal endarteritis. Similar histological alterations were observed by O'Hare and associates (110). Page (111) found that hypertension secondary to irradiation of the kidneys is not abolished by renal denervation.

Pedersen and Bell (112, 113) produced hypertension in rabbits by constricting the renal vein with an aluminum band and by placing a membrane around the kidney in order to prevent the development of venous collateral circulation. These findings were confirmed by Menendez (114) on dogs.

Compression of the kidneys by an oncometer resulted in a slight elevation of the blood pressure in acute experiments by Alwens (115). Loesch (116) found a moderate persistent elevation of blood pressure in dogs in which intermittent brief occlusion of the renal arteries, veins and

ureters was produced repeatedly. Page (117) reported recently that arterial hypertension can be produced in dogs by placing cellophane around one or both kidneys. The pressure rises after several weeks and usually remains elevated. Denervation of the kidney does not interfere with the development of hypertension. At autopsy, the kidney is found to be surrounded by a thick shell of scar tissue.

VI. *Constriction of renal arteries (renal ischemia).* a. *Methods of production.* Katzenstein (118) in 1905 noted a slight elevation of the arterial pressure in acute experiments on dogs following partial occlusion of the renal arteries. Halsted (119) partially occluded one of the two renal arteries with an aluminum band but did not determine the blood pressure. In acute experiments on dogs, Bridgman and Hirose (120) found no alteration of the blood pressure following the constriction of one renal artery by the aluminum band of Halsted and ligation of the opposite renal artery. They stated, "A similar study of animals in whom a constricting band had been left for a considerable period around the renal artery, simulating a chronic lesion, would be of interest, but external events prevented our undertaking it, as had been hoped." Drury (121) in 1932 described briefly a method for the production of renal insufficiency. This consisted of constricting by a silk ligature one renal artery of young rabbits. The diameter of the loop placed about the left renal artery was regulated by tying the silk ligature down on a wire of known diameter. When the animal attained its growth, the opposite kidney was removed. Depending upon the amount of constriction of the renal artery as produced by the ligature, he found that any desired degree of renal insufficiency could be produced. The blood pressure was not determined in these experiments. Recently (1938), further studies using this method were reported by Drury (96). Renal insufficiency of any desired degree was produced in rabbits without resulting pathologic changes in the kidney epithelium. A moderate elevation of the blood pressure was observed prior to the removal of the non-ischemic kidney and it became more marked following nephrectomy.

The fundamental work of Goldblatt and his associates (46) on renal hypertension was begun in 1928. Although it had been suggested that renal ischemia might play an important part in the development of human hypertension, there was no good experimental proof for this deduction. With the working hypothesis that ischemia limited to the kidneys might be the initial condition in the pathogenesis of some types of hypertension, Goldblatt, Lynch, Hanzal and Summerville (46) de-

vised a silver clamp with which the degree of constriction of the main renal artery could be varied and controlled. They found that constriction of one renal artery was followed by a moderate rise of blood pressure which usually returned in several weeks to the level of the control period. Marked constriction of both renal arteries resulted in a marked elevation of the systolic blood pressure which was accompanied by a severe disturbance of renal function and uremia. Moderate constriction of both renal arteries resulted usually in a persistent elevation of the systolic blood pressure. Most interesting is the fact that this condition was unaccompanied by signs of decreased renal function. Findings similar to those noted in dogs were found in experiments (122) on monkeys. It was necessary in some instances to increase the constriction in order to maintain an elevation of the blood pressure which had declined as accessory circulation to the kidney developed. Wood and Cash (97) and Goldblatt found that bilateral renal ischemia resulted in a persistent elevation of the diastolic as well as the systolic pressure. Thus the handicap to the study of hypertension which resulted from inability to produce experimentally with great regularity a persistent and marked elevation of pressure in dogs and monkeys such as one encounters in patients seems to have been removed by the ingenious method of Goldblatt. Mason, Evers and Blalock (123) found that hypertension produced in this manner is not accompanied by an alteration in the renal arteriovenous difference in oxygen content. Levy, Light and Blalock (79) measured the renal blood flow and blood pressure distal to the constriction in animals with persistent hypertension and found both of these functions as well as the oxygen consumption to be decreased.

b. *Pathologic findings.* The pathologic findings in animals following the production of renal ischemia have been described by Goldblatt (46, 124), by Wilson and Pickering (125), by Elaut (126) and by Child (127). When the constriction of the renal arteries was made severe at the beginning, the resulting elevation of blood pressure and uremia were associated with the development of fibrinoid and hyaline degeneration and necrosis of arterioles, with petechiae in some of the organs. The lesions are similar to those observed in the acute malignant phase of essential hypertension in man. The acute arteriolar lesions were most marked in the vessels of the intestinal tract. Goldblatt (124) considers the acute arterial lesions to be the results of the combination of the hypertension and the renal insufficiency. On the other hand, if the constriction of the renal arteries was less severe and resulted in

long standing hypertension without demonstrable damage of renal function, the small arteries and arterioles show thickening of the media and sometimes slight hyalinization of the intima, especially in the retinal arterioles. The changes in the kidneys are mainly in the tubules. Perhaps the most interesting point is that renal ischemia and the associated hypertension result in less damage to the renal arterioles than to the arterioles of most other organs with the exception of the lungs. The pressure in the pulmonary vessels is not increased (128). These findings suggest strongly that the increased intravascular pressure is responsible for the alterations in the vessel walls because, as has been stated, the pressure in the intrarenal vessels is prohibited from rising greatly by the constricting action of the clamp. The recent findings of Wilson and Byrom (129) in experiments on rats in which hypertension was produced by occlusion of one renal artery support this viewpoint. Renal vascular lesions were limited to the non-ischemic kidney. In essential hypertension of patients, the main renal arteries are usually not constricted and the most marked damage is found in the arterioles of the kidneys. Wilson and Pickering (125) found that the severity and extent of the experimental lesions bear a fairly close relationship to the degree of hypertension rather than its duration. It is of significance that the most severe lesions observed by Goldblatt have been in animals that have had a fairly long period of hypertension preceding the induction of renal insufficiency caused by further tightening of the clamps. In such animals in addition to the acute changes, thickening of the media with or without hyalinization of the intima of the arterioles was found. Rupture of the aorta with resulting cardiac tamponade occurred in one of the animals of Blalock, Levy and Cressman (130). Keyes and Goldblatt (131) have studied the eyes of dogs and monkeys in which hypertension had been present for more than five years. Changes similar to those seen in man with benign and malignant essential hypertension were observed. Concentric hypertrophy of the left ventricle was found by Elaut (126). Finally, Moritz and Oldt (132) have presented additional histological evidence suggesting that renal arteriosclerosis is the primary lesion in essential hypertension in man.

c. *Mechanism of production.* That ischemia of a kidney or kidneys is responsible for the elevation of pressure has been demonstrated by a number of experimental procedures. As has been stated, bilateral nephrectomy does not result in hypertension. Release of the arterial constriction or removal of the ischemic kidney (47, 76, 133), the other

kidney being normal, causes an elevated pressure to return to normal in approximately six hours (76). The release of bilateral renal clamps also results in a decline of pressure to the control level. Constriction of the arterial supply to a single transplanted kidney results in an elevation of blood pressure and release of the constriction is followed by a decline (76, 134, 135, 136). Further evidence is furnished by the results of experiments in which the aorta is constricted. Rytand (137) and Goldblatt, Kahn and Hanzal (138) found that constriction of the abdominal aorta just above the site of origin of both main renal arteries results in hypertension whereas constriction of the aorta below the renal vessels has no significant effect on the blood pressure. Steele (139) recently reported that clamping of the aorta above the orifices of the renal arteries in dogs is followed by an elevation of the diastolic level of arterial pressure in the femoral as well as in the carotid arteries. Longcope and McClintock (140) constricted the coeliac axis and the superior mesenteric artery of dogs and found no significant alteration in the blood pressure. Goldblatt (46) found that constriction of the splenic and femoral arteries did not result in a rise of blood pressure. Blalock and Levy (43), by multiple stage operations, produced complete occlusion of the coeliac axis, the superior and inferior mesenteric arteries. A temporary elevation of blood pressure occurred which in some instances did not quite return to the control level, but the elevation was not marked. Thus it would seem certain that ischemia of the kidneys is responsible for the hypertension under consideration. As stated, there is a decrease in the renal blood flow and arterial pressure distal to the point of constriction (79).

The mechanism whereby renal ischemia results in hypertension has been the subject of intensive investigation in the past few years. The control of the arterial pressure is chiefly dependent upon the peripheral resistance, the cardiac output and the blood volume. Freeman and Page (141) found that the production of hypertension by the Goldblatt method does not cause an increase of the plasma volume and Holman and Page (142) observed no alteration in the cardiac output. There is no evidence for an increase of blood viscosity. It would appear then that the hypertension is due to resistance offered to the flow of blood in the finer divisions of the arteries, the arterioles. As Cannon (143) has stated, there may be three explanations of the hypertensive state of the arterioles; 1, excessive discharge of vasoconstrictor impulses from the central nervous system, such as to induce an abnormal narrowing of the channels; 2, increased sensitiveness of the smooth muscle

of the arterioles to natural stimuli which cause contraction, or 3, pathologic constriction of the small vessels because of direct action upon them of unusual chemical agents. There is very strong evidence that hypertension associated with renal ischemia is not produced through a nervous mechanism. It is not prevented or abolished by denervation of the kidneys (111, 144, 126, 145), by section of the splanchnic nerves and excision of the lower four thoracic sympathetic ganglia (146), by subdiaphragmatic splanchnicectomy and removal of the coeliac and upper lumbar ganglia (76), by section of the anterior spinal nerve roots from the fifth thoracic to the third lumbar (147), and by excision of the entire sympathetic chain in the abdomen and chest including denervation of the heart (141, 148, 145). Inconclusive results were obtained in sympathectomized dogs by Alpert, Alving and Grimson (149). Glenn, Child and Page (150) found that destruction of the spinal cord below the fifth cervical vertebra in dogs with hypertension produces an immediate sharp fall of pressure, which is followed by a rise to above the normal but never to the hypertensive level. Glenn and Lasher (151) state that the production of renal ischemia in dogs in which the spinal cord had been destroyed below the fifth cervical vertebra resulted in hypertension. Finally, as has been stated, constriction of the blood supply of a kidney which has been completely denervated by removing it and transplanting it to another part of the body results in hypertension (76). All of these observations seem to prove that a nervous reflex from the ischemic kidneys is not the mechanism responsible for the initiation of the hypertension. As Cannon (143) has stated, there is the bare possibility that the hypothetical agent might influence primarily prevertebral ganglia such as the coeliac and the inferior mesenteric, and by exciting these isolated groups of cells which constrict the splanchnic vessels, might be the occasion of the hypertensive state.

There is no convincing evidence of increased sensitiveness of the smooth muscle of the arterioles to natural stimuli which cause contraction. Bouckaert, Elaut and Heymans (152) reported an increase of the reflex excitability of the vasoconstrictor mechanisms in dogs with renal hypertension. This finding was not confirmed by Verney and Vogt (145). The latter authors noted that sensitivity of hypertensive dogs to injected adrenalin was usually normal, while that to tyramine was often increased. They do not believe hypertension of renal origin to be due to an increased concentration of tyramine in the blood. Rogoff, Marcus and Wasserman (153) have shown that there is no increase

of epinephrine secretion in hypertension due to renal ischemia. Goldblatt and co-workers (46) found that extirpation of one entire adrenal and the medulla of the opposite one did not prevent the elevation of pressure associated with renal ischemia. It was reported by Goldblatt (47), by Blalock and Levy (76) and by Page (154) that bilateral extirpation of the adrenals prevents the rise of blood pressure or causes it to decline if it is already elevated. It is necessary to leave only a small portion of the adrenal cortex for the blood pressure to rise and remain high. Levy and Blalock (155) found that removal of one adrenal and denervation of the other by transplanting it to the neck does not prevent the development of or abolish renal hypertension. In most of the bilaterally adrenalectomized animals of Goldblatt (47), of Page (154) and of Collins and Wood (156), moderate hypertension developed when adequate supportive and substitution therapy was given. Collins and Wood (156) found that the blood pressure of adrenalectomized dogs with renal ischemia declined but did not reach the average normal level even in periods when no cortical extract was given. Enger, Linder and Sarre (157) found that the production of renal ischemia in adrenalectomized dogs resulted in a moderate temporary elevation of the blood pressure. Fasciolo (158) has shown that grafting the ischemic kidney of a dog with hypertension into a chloralosed nephrectomized dog produces a rise of pressure even if the adrenals have been removed. Finally, Rogoff, Nixon and Stewart (159) have found that this type of experimental hypertension may exist in bilaterally adrenalectomized, untreated dogs. Regardless of whether or not hypertension associated with renal ischemia can be induced in adrenalectomized animals, it seems unlikely that the adrenal is implicated specifically in its etiology other than in the sense that the adrenal cortex is important in the maintenance of the blood pressure in all conditions including the normal. Studies have also been performed on the relationship of other endocrine glands to the development and maintenance of renal hypertension. Page and Sweet (160) and Page (154) found that the effect of removal of the hypophysis is to dampen rather than to abolish the effects of renal ischemia. These findings were confirmed by Enger, Linder and Sarre (157). This type of hypertension is not abolished by total thyroidectomy (161) or removal of the ovaries or testes (154). The prevailing evidence indicates that the endocrine glands are not essential for the development of hypertension associated with renal ischemia but that they are important in determining the degree of response.

Since experimental hypertension associated with renal ischemia is

not due to an increase of blood volume or cardiac output, or dependent upon a decrease of the excretory functions of the kidneys, since it is not produced through a nervous mechanism and since the evidence indicates that the endocrine hormones are not directly concerned in its pathogenesis, one is more or less reduced to the possibility that renal ischemia results in hypertension through a chemical mechanism, probably through the release into the circulation of a pressor substance. Supporting this view is much evidence such as the fact that occlusion of the renal vein in the presence of constriction of the renal artery, thereby eliminating the possibility of a hypothetical pressor substance entering the circulation, does not result in hypertension (47). Also interesting is the fact that an ischemic kidney may result in at least a temporary elevation of the arterial pressure in the presence of a normal non-ischemic kidney. But perhaps the most convincing evidence for a humoral factor that has been obtained thus far is that which has resulted from excluding other possible mechanisms.

Renal pressor substance. The recent work on experimental hypertension has focused attention upon the description by Tigerstedt and Bergman (162) in 1898 of a renal pressor substance. This was found in saline extracts of fresh rabbits' kidney or in the dry residue obtained after treating rabbits' kidney with alcohol. This substance which was named "renin" was obtained in the main from the cortex of the kidney. It was described as being non-dialysable, stable at 56° but destroyed by boiling, soluble in water and dilute salt solutions, insoluble in acetone and in 50 per cent alcohol. Renin when injected into anesthetized rabbits caused an elevation of blood pressure which lasted as long as 20 minutes. The observations of Tigerstedt and Bergman indicated that the pressor effect of renin is due to a peripheral action and that it is excreted partially through the kidneys. Some of the workers in this field have confirmed the findings of Tigerstedt and Bergmann, others have obtained only depressor effects while the common finding has been that of a combination of pressor and depressor effects. Collip (163) has stated that extracts of other organs may exert pressor effects. Pickering and Prinzmetal (164) state that the pressor action of renin may be reduced or abolished by anesthetics such as urethane, nembutal and ether. Some of the conflicting results may be explainable on this basis. The purest preparation of Bingel and Strauss (165) was obtained from autolysed renal press juice by fractional precipitation with ammonium sulphate. The findings of Bingel and Strauss (165), Hartwich and Hessel (166), Hessel and Maier-

Hüser (167), Pickering and Prinzmetal (164), and of Landis, Montgomery and Sparkman (168) indicate that renin is either a protein or protein-like substance. The findings of Kohlstaedt, Helmer and Page (169) suggest that renin is an enzyme-like substance which is activated by a kinase-like material contained in the protein fraction of plasma and whole blood. Improvements in methods for preparation and purification of renin have been described by Grossman (170) and Helmer and Page (171). The latter authors state that renin prepared by their method is stable for at least two months when kept cold. Landis, Montgomery and Sparkman (168) have found that the pressor activity can be made more consistent by appropriate heating. Furthermore, they made the important observation that peripheral blood flow is not reduced during the period when the blood pressure is elevated. Other pressor substances were studied and the specially treated saline kidney extract was the only one which elevated peripheral blood pressure without simultaneously reducing skin temperature in the ear of the warmed rabbit. This property of renin suggests a mechanism similar to that of hypertension in man but it differs in that the pressor effect of renin is not sustained more than 45 minutes and subsequent repeated injections yield smaller responses. Landis, Jeffers and Shiels (172) have found that heating the extract to 55° precipitates at least some of the pressor substance in addition to diminishing the depressor effects.

Williams, Harrison and Mason (173) showed that the pressor response of renin is not abolished by cocaine as is that of tyramine. Two different pressor substances were obtained from extracts of renal tissue. Williams and Grossman (174) obtained two pressor substances by perfusion of isolated kidneys of hogs and dogs. One of these substances resembled Tigerstedt's renin in its properties while the other was believed to be adrenalin or some adrenalin-like substance. The pressor effect of the latter substance was enhanced by cocaine and diminished by ergotamine. Williams (175) noted subsequently that the rise of blood pressure in white rats produced by renin is enhanced by cocaine and inhibited by ergotamine. Since adrenine is similarly affected, Williams suggested that the prolonged rise of pressure with renin injections is due to gradual liberation of an adrenine-like substance. Hessel (176), Friedman, Abramson and Marx (177) and Helmer and Page (171) have found that the pressor action of renin is not abolished by ergotamine nor potentiated by cocaine. The contradictory results may be due to the use of different test animals or to the presence of

impurities in the extracts. Boylston, McEwen and Ivy (178) were unable to obtain a pressor substance in significant amounts from the ischemic kidneys of hypertensive dogs by perfusing them with Locke's solution. No significant differences in the vasoconstricting properties of normal plasma and of plasma from hypertensive dogs on arterial rings were found by Wakerlin and Yanowitz (179).

Grossman and Williams (180) found that the kidneys of young rats contain a greater quantity of renal pressor substance than those of old rats, and that the blood pressure response of old rats to injection of the renal pressor substance is greater. It was noted by Friedman et al. (181) that ablation of the adrenal glands was followed by a progressive diminution in response to the renal pressor substance but not to other vasoconstrictor substances. The sensitivity to renin returned when cortin was given. Similar results were obtained by Williams, Diaz, Burch and Harrison (182). Also it was noted that the kidneys of adrenalectomized rats usually contain more of the renal pressor substance than do those of normal rats.

Regarding the site of action of the renal pressor substance, Hessel and Maier-Hüser (167) stated that the vessels of the extremities, intestines and kidneys are constricted. Merrill, Williams and Harrison (183) observed that the injection of renin caused a rise of blood pressure after destruction of the spinal cord and after exclusion of the hypophysis, adrenals, pancreas, liver and kidneys from the circulation. The same authors confirmed the observation of Tigerstedt and Bergman (162) that animals which had been subjected to nephrectomy several days previously exhibited a marked increase in sensitivity to renin. Tigerstedt and Bergman ascribed this difference to failure of excretion of renin by the nephrectomized animal. However, Merrill et al. (183) found that this increase in sensitivity did not develop immediately after nephrectomy but appeared one or more days later. This observation led Grollman, Harrison and Williams (184) to suspect that normal renal tissue might form some substance capable of antagonizing the action of renin. A method for the bio-assay of renin in which nephrectomized dogs are used was described by Wakerlin and Chobot (185). Leiter and Eichelberger (186) observed that the injection of renin into dogs with renal ischemia produced a more prolonged pressor response than was found in normal dogs. A vasoconstrictor effect was demonstrated (183) on perfusion of the isolated leg. Similar findings were obtained by Friedman, Abramson and Marx (177). Subsequent experiments by Merrill et al. (187) indicated that the kidney is par-

ticularly sensitive to the action of renin as the diminution of renal blood flow was more pronounced than the elevation of blood pressure. The results of these and other experiments suggested to the authors that renin produces contraction of the efferent glomerular vessels. Similar results were obtained by Corcoran and Page (188). On the other hand, Steele and Schroeder (189) found that the injection of renin caused an increase of the renal blood flow.

Harrison and his associates (83, 190), Prinzmetal and Friedman (191) and Govaerts and Dicker (192) found that extracts of kidneys of dogs with hypertension exerted greater pressor effects than those of control kidneys. Comparison of the extracts prepared from the two kidneys of dogs rendered hypertensive by compression of one renal artery showed a greater pressor effect from the abnormal than from the normal kidney. Increased pressor properties in the blood of dogs with renal ischemia and hypertension could not be demonstrated by Prinzmetal, Friedman and Rosenthal (193), by Page (194), by Collins and Hoffbauer (195) and by Heymans and Bouckaert (21). Dicker (196) states that the blood of hypertensive dogs contains pressor substances which are absent in normal dogs. This observation would appear to need confirmation. Houssay and Taquini (197, 198, 199) made comparative studies of the vasoconstrictor action of the plasma of venous blood from both normal and ischemic kidneys, the Laewen-Trendelenburg method on the toad being used. They found in all instances that the venous blood from an ischemic kidney causes greater vasoconstriction than that from a normal kidney or any other organ. Further, it was noted that the venous blood from the normal kidney of an animal with unilateral renal ischemia contains less of the pressor substance than the arterial blood, indicating that the normal kidney destroys this substance. Mason (200) has been unable to confirm these observations on North American toads and bull frogs using the method employed by Houssay and Taquini. In connection with this general problem, it is of interest that Verney and Vogt (145) found in experiments in which an isolated kidney and a loop of small intestine were each perfused by a heart-lung preparation that the perfused kidney liberates a substance which produces vasoconstriction in the gut. Wakerlin and Chobot (201) state that their experiments yield no evidence for the possible rôle of renin in the maintenance of normal blood pressure.

Although there is no conclusive proof of the existence of a known or new pressor substance in the blood, spinal fluid or urine in experi-

mental hypertension due to renal ischemia, the findings suggest that the increase of blood pressure is dependent on the excessive formation of a pressor agent which is also present in normal kidneys. As Harrison (190) has stated, other possibilities are that the substance formed in the kidney with a defective blood supply might be somewhat different in composition and more active pharmacologically, that the kidney with an impaired circulation is less capable of excreting a normal quantity of pressor substance, that the pressor agent may be some intermediary metabolic product and the reaction fails to go on to completion in the absence of a normal blood supply, and lastly that the occurrence of increased pressor activity in the ischemic kidney may bear no relationship to the rise of blood pressure. A further remote possibility is that there is a decrease of depressor substances rather than an increase of the pressor ones.

As has been stated, the hypertension which results from constriction of the blood supply to one kidney only usually does not persist. The fact that it occurs at all indicates that the hypothetical agent is one which the normal kidney cannot excrete rapidly. The fact that the hypertension does not persist indicates that the normal kidney is a factor in determining the response to renal ischemia. Further evidence which supports this was found by Blalock and Levy (76) in experiments in which removal of the non-ischemic kidney resulted in hypertension in animals with unilateral ischemia and a normal blood pressure. Similar results have been obtained by Katz, Mendlowitz and Friedman (202), by Verney and Vogt (145) and by Fasciolo (199). Rodbard (203) states that the destruction, neutralization or elimination of the chemical mediator can be accomplished at a rapid rate only in the presence of kidney tissue. Evidence that the normal kidney is capable of reducing the rise in pressure produced by the ischemic kidney is in keeping with the experiments of Grollman, Harrison and Williams (184) in which it was found that a substance which lowers the blood pressure of hypertensive rats (subtotal nephrectomy) when administered orally can be obtained from the kidneys of hogs. The conception that normal renal tissue produces something capable of lowering a pathological elevation in the blood pressure is not supported by the fact that an occasional dog with only unilateral renal ischemia will have a persistent elevation in the blood pressure. Furthermore, it has been found by Blalock, Levy and Cressman (130) that a high percentage of animals with unilateral renal ischemia combined with intestinal ischemia, produced by the gradual complete occlusion of the

coeliac axis, the superior and inferior mesenteric arteries, will have a sustained elevation of blood pressure.

Attempts to modify the hypertension (renal ischemia). Many attempts to influence the hypertension associated with renal ischemia have been made in addition to those described already in connection with operations on the nervous system and the endocrine glands. Cash and Wood (204) found that diets which cause a marked gain in weight in dogs with renal hypertension result in a marked elevation of the systolic blood pressure. Reduction of weight in these animals was associated with a decline of the systolic but not of the diastolic pressure. Verney and Vogt (145) found that increased dietary loads elicited a reversible rise of the blood pressure level of hypertensive dogs with renal ischemia, sodium chloride being more effective in this respect than urea or meat. They state that the sensitivity of the arterial pressure to changes in dietary load is dependent upon renal ischemia and not upon a simple reduction in the quantity of functioning renal tissue. Particularly interesting are the preliminary observations of Dill and Erickson (205) that an eclampsia-like syndrome occurs in pregnant dogs and rabbits when the renal arteries are constricted. On the other hand, unpublished observations by Goldblatt, by Grollman, Harrison and Williams and by Dawson, Cressman and Blalock show that there is a decline in the pressure in hypertensive dogs and rats during the terminal part of the pregnancy period.

Attempts to prevent the development of or to abolish hypertension associated with renal ischemia have in the main been unsuccessful. The passage of the venous blood returning from the kidneys through the liver (206, 207) does not affect this type of hypertension. Previous damage to the kidneys, partial constriction of the renal veins and hyperpyrexia are without influence (207). Levy and Blalock (207) noted that a severe illness such as distemper is usually accompanied by a decline of the elevated blood pressure, which is another point in common with hypertension as observed in man. Preliminary experiments by Davis and Barker (208) indicate that the decline of blood pressure following the giving of cyanates to dogs with renal ischemia is usually greater if a splanchnicectomy has been performed. As Goldblatt (209) has stated, one obvious procedure which suggests itself in clinical or experimental hypertension is the possible improvement of blood supply to the functioning components of the kidney by attempts to increase the collateral circulation. He stated, "If, before constricting the renal artery, the kidney is decapsulated and

adipose tissue or muscle is attached to the denuded cortical surface, the accessory circulation becomes very prominent and interferes with the development of pronounced elevation in blood pressure." MacNider and Donnelly (210) and Davis and Tullis (211) have reported results which indicate that an accessory blood supply to the normal kidney may be created artificially. Mansfield, Weeks, Steiner and Victor (212) found that the hypertension produced by constriction of the renal arteries is lowered by pexis between the kidney and the omentum or spleen. It was observed that the reduction in blood pressure with the omental union was only temporary whereas that with the kidney-splenic union was sustained. It is difficult to draw conclusions from such experiments, as is demonstrated by the fact that animals may survive complete occlusion of both main renal arteries (47, 76) when it is effected gradually without any attempt being made to create a new collateral blood supply. Further, the employment of such a procedure in the treatment of human hypertension would be rarely indicated because the vascular disease most frequently includes involvement of the preglomerular arteries. In the instances of sclerosis of the larger renal vessels, an attempt to cause an increase of the collateral blood supply might result in benefit, but even this is very doubtful. The results (213) of nephro-omentopexy in man are not very encouraging. Unfortunately, no means has as yet been devised to make a kidney survive following homo-transplantation.

SUMMARY (RENAL ISCHEMIA)

In concluding this consideration, it may be stated the evidence indicates that diminution in the renal blood flow produces peripheral vasoconstriction and hypertension by means of the action of a pressor substance which is formed in the ischemic kidney and which enters the blood stream. The exact nature of the product elaborated by the kidney has not been identified. It is likely that it is produced in small amounts over a long period of time and it is possible that its identification is not possible by the methods available at present. The most hopeful line of investigation from the therapeutic viewpoint lies in the possibility of finding a specific anti-pressor substance. An interesting application which has already resulted from this work consists of the disappearance of hypertension following nephrectomy in patients with unilateral pyelonephritis and vascular disease (214–218).

It is not believed that renal ischemia is responsible for all instances of experimental hypertension. Katz (202) is of the opinion that hyper-

tension of renal origin depends on the ratio of ischemic to normal kidney tissue rather than on the amount of ischemic renal tissue alone. Some believe that ischemia is only one of many abnormalities that may result in hypertension. However, it is interesting that the renal blood flow is known to be reduced in hypertension produced by the Goldblatt method and in that due to ureteral occlusion; it is almost certainly reduced in coarctation of the aorta and in the chronic form of experimental glomerulonephritis and the total renal blood flow is diminished in hypertension due to a reduction in the amount of functioning renal tissue as accomplished by various methods even though the evidence indicates that it is not reduced per gram of kidney tissue; it may possibly be reduced in hypertension due to the injection of kaolin into the cerebrospinal system and in that due to section of the moderator nerves, the evidence for this being contradictory, some claiming that denervation of the kidneys abolishes the hypertension. Many of the observations that have been enumerated probably have a distinct bearing on clinical hypertension. Karsner (219) stated recently, "The observations in experimental animals and in man give no support to the view that essential hypertension is different from renal hypertension.... Failure to demonstrate any signs of renal incompetency in life is not indicative of the absence of arteriolar disease of the kidneys.... The burden of proof must be with those who make a qualitative classification of forms of chronic hypertension in opposition to the view here expressed that the disease is basically unitary."

Granting that there are some types of hypertension that are non-renal in origin, the evidence which has been reviewed indicates that most instances of experimental and probably of clinical hypertension are related to some abnormality in function of the kidneys.

ADDENDA

A number of important articles on experimental hypertension have appeared since the completion of this review. No attempt will be made to refer to all of them. Particularly outstanding among the recent contributions are the following.

1. Goormaghtigh and Grimson (220) have presented evidence in support of the existence of an endocrine gland in the wall of the renal arterioli and are of the opinion that it is principally the afibrillary muscle cells of the media of the arterioli which are the endocrine elements. Goormaghtigh (221) states, "Renal ischemia causes hypertrophy and multiplication of certain cells of the media of the renal

arterioli which normally lack myofibrillae and which in the rabbit possess the cytologic characteristic of endocrine cells. Provided it is fairly pronounced it causes the transformation of muscle cells with myofibrillae into afibrillary cells which always become granular in the rabbit and sometimes, though more discretely so, in the dog. If ischemia lasts for several months many afibrillary renal arteriolar elements in the dog become granular and acquire the cytologic endocrine characteristics seen in the rabbit." The most obvious objection to this interpretation is that the arterioli of all organs are said to contain afibrillary cells and it is known that progressive constriction of the arterial supply of organs other than the kidneys does not result in hypertension. Goormaghtigh (221) states that this difference is due to the fact that the afibrillary cells of the various organs are not identical and that in no organ except the kidneys is there such close contact between the cells of the arteriolar media and the cells of the parenchyma.

2. Page (222) has recently described the results of a large number of experiments bearing upon the mechanism of renin tachyphylaxis (decreased pressor response to consecutive injections). It has been shown previously that purified renin, when incorporated in Ringer solution and used as a perfusate for isolated organs, exhibited no vasoconstrictor action, presumably because of the absence of a protein activator present in normal blood (169, 223). The pressor action is restored by the addition of partially purified activator, or by addition of blood.

The isolated rabbit's ear exhibits tachyphylaxis to renin, which is abolished by the addition of renin activator, and the phenomenon in the isolated organ appears to be due solely to exhaustion of the supply of activator in the perfusate. In the intact tachyphylactic animal, however, the pressor effect of renin is not restored by addition of activator alone, and Page concludes that the refractory state is the consequence of two phenomena, (a) exhaustion of renin-activator, and (b) the appearance of an anti-pressor substance, or the development of an "anti-pressor state."

The hypothetical anti-pressor substance was not directly demonstrated. As tachyphylaxis was not studied in nephrectomized animals, it is not clear whether this anti-pressor substance is related in any way to that claimed to be found in and formed in kidneys by Grollman, Harrison and Williams (184, 224).

More recently Page and Helmer (225) have studied the interaction of renin and renin activator and state they have isolated the resulting product as the crystalline oxalate and picrate. This substance, desig-

nated "angiotonin" is water soluble, heat-stable, dialyzable, fluorescent, and gives a positive Sakaguchi reaction. It is strongly pressor with a somewhat more prolonged effect than adrenalin, its action being primarily upon the peripheral blood vessels. Only moderate tachyphylaxis is observed following repeated injections into cats and dogs. The authors believe "angiotonin" is an intermediate product of the interaction of renin (enzyme) and renin-activator (substrate) as prolonged contact between the two reduces the yield of pressor substance. It is their view that renin is an enzyme, contained in the kidneys, and without pressor properties, which reacts with activator in blood to form "angiotonin," a pressor substance which may provide the body with a humoral means of regulating arterial pressure.

3. Harrison, Grollman and Williams (226) have found that the blood pressure of dogs with hypertension due to renal ischemia may be reduced by the administration of their renal anti-pressor substance. This extract which also reduces the blood pressure of rats with hypertension due to subtotal nephrectomy does not cause a decline in the blood pressure of normal rats (184, 224). Some of their animals have become seriously ill while under treatment and this finding has delayed study of this form of therapy for patients. Although a few patients have been treated with encouraging results, the authors state that their extract is not yet sufficiently free from impurities to be ready for general clinical trial (226).

4. Recent studies of the respiratory metabolism of tissue slices from normal and hypertensive kidneys have yielded conflicting results (227, 228).

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PLASMA PROTEINS: THEIR SOURCE, PRODUCTION AND UTILIZATION

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The subject of this review is one generally shunned by texts on physiology and perhaps this is evidence of wisdom on the part of the authors concerned. Having accepted the responsibility for this review the writers must expose their ignorance, and perhaps that of other workers in this field, and invite critical comment. It will give us both pleasure if this review is helpful to co-workers in this difficult but rapidly expanding field. We express regret if lack of space and time has led to oversight of significant contributions.

It may be stated with confidence that the keen interest in amino acids as relating to protein digestion and utilization in the body actually was responsible for the view that plasma proteins were inert substances having little or nothing to do with tissue nutrition and internal nitrogen metabolism. The evidence now appears convincing that under certain conditions (and probably more or less continuously) there is a "give and take" between body proteins and plasma proteins. For example, a dog while fasting can be maintained in nitrogen equilibrium by plasma protein given intravenously.

The chemical and physical natures of proteins have received much attention in recent years but a review (74) of this knowledge does not yet allow us to describe definitely the proteins under consideration. Although recent evidence from Scandinavian, British and American laboratories (reviewed in (74)) renders it probable that these plasma protein fractions are all part of a single, variably bound plasma protein system, we shall continue to speak of *albumin*, *globulin*, and *fibrinogen*, for they do have a certain *independent importance* in biological reactions. Moreover, to be acceptable new hypotheses of protein structure cannot be incompatible with this independence.

SITE OF FORMATION OF PLASMA PROTEIN. It is our conviction that the liver is of primary importance in the production of plasma proteins but it must be admitted that there is much conflicting evidence in the literature (51, 67, 7).

There is much evidence that *fibrinogen production is wholly dependent upon liver function.* Many experiments with hepatic poisons (chloroform and phosphorus) (18, 22, 64, 88, 29, 75) give irrefutable evidence that when the liver is injured the blood fibrinogen falls rapidly and somewhat in proportion to that liver injury. With liver regeneration and repair the fibrinogen blood values return to normal. Injury to any other tissues (in the absence of liver injury) is followed by *elevation* of the blood fibrinogen. Such experiments are not absolutely conclusive (as are very few observations in physiology) but they cannot be put aside by critics until more convincing experiments pointing to a different interpretation are forthcoming.

Hepatectomized animals give supporting evidence but on the whole no more convincing than the experiments with liver injury. The relatively short life after liver removal and the profound systemic and circulatory disturbances are factors which confuse the picture after hepatectomy (90, 53, 52, 72, 36). The most satisfactory hepatectomy experiments relating to fibrinogen are those of Drury and McMaster (23) and Jones and Smith (36). In dehepatized rabbits (23) a drop in blood fibrinogen was recorded—35 to 65 per cent below normal in 15 to 30 hours. In dogs (36) there was a definite and progressive fall in fibrinogen to 16 to 47 per cent below the control levels within 13 to 20 hours after hepatectomy. It must be admitted that we do not know how rapidly fibrinogen is used in the normal and particularly in the abnormal animals and this lack of knowledge weakens the deductions drawn from the hepatectomy experiments.

One who is acquainted with the literature hesitates to bring up the subject of the *origin of albumin and globulin.* In the case of fibrinogen the opinions center more or less about the liver but in the debates about the source of albumin and globulin hardly an organ or tissue escapes, unless it be the brain. And in fact it may be that under certain circumstances almost any tissue can contribute to a globulin fraction. We prefer to state our own convictions first and submit arguments relative to these beliefs before mentioning some of the other views. The present evidence speaks in favor of the liver as the site of production of albumin and of much of the globulin. It must be admitted that many proteins appearing in the globulin mixture may be derived from body cells other than the liver.

The evidence pointing to the liver as the site of albumin and globulin formation is both clinical and experimental. Some years ago Kerr, Hurwitz, and Whipple (39) noted a definite lag in the regeneration of

serum protein of Eck fistula dogs following acute plasma depletion. More recently Knutti, Erickson, Madden, Rekers and Whipple (40) reported observations over two years on an Eck fistula dog. Always in excellent clinical condition, this dog at times was unable to form new plasma protein in significant amounts on various standard diets; in fact, on occasions this dog could form only about one-tenth as much plasma protein as normal controls. At autopsy, the only significant findings were a perfect Eck fistula and the usual atrophic liver found in these dogs.

Hepatectomy experiments (15, 27) have added little significant evidence relating to plasma albumin and globulin. Partial hepatectomy in rats (17) is followed by depression of plasma protein within 24 hours. It would be interesting to know something about blood volume at this time in these rats. The albumin fraction remains low for the subsequent 4 weeks and this observation may be significant. Moreover, Warner, Brinkhous and Smith have found that either partial hepatectomy or chloroform injury results in a marked decrease in the plasma prothrombin, an important one of the many globulins (81, 80).

Clinical observations point to the liver as the source of plasma proteins (14). A child with edema and *hypoproteinemia* was observed for 7 months and at autopsy Thompson, McQuarrie and Bell (77) observed liver atrophy with disappearance of cells in intermediate and peripheral zones in the lobules. The other tissues were normal. Johansen (35) added a case of idiopathic hypoproteinemia which at autopsy showed an *interstitial hepatitis*. Cases of liver cirrhosis often show a low level of plasma protein, especially a low albumin:globulin ratio.

One almost perfect experiment is frequently overlooked by physiologists who regard the bone marrow as the source of plasma proteins. Human cases of *aplastic anemia* do come to autopsy with almost complete absence of all marrow elements yet with *plasma proteins all* within normal range. This would seem to dispose of the marrow cells (7) as an essential factor in plasma protein fabrication.

Hyperproteinemia has been recorded in a variety of clinical diseases—myeloma (12), osteomyelitis, lymphogranuloma inguinale (89), syphilis. The globulin fraction is usually responsible for this increase and evidence (12) has been submitted to show the presence of an abnormal globulin. Qualitative changes in the serum proteins in renal disease (nephrosis) have been found by immunological (30) and precipitation experiments (34).

Perfusion experiments are difficult to evaluate (11, 60) but may pre-

sent intriguing data (19). Cutting and Cutter (19) found that injection of india ink abolished the ability of the rat to replace acute loss of plasma protein. We do not regard the reticulo-endothelial system as major factor in plasma protein formation but a growing body of evidence, such as that offered by Sabin (73), indicates that some globulin can be made in body cells outside of the liver. Arguments pro and con for a transformation of albumin into globulin or vice versa cannot be reviewed at this time (61, 28, 82, 24, 7).

Materials concerned in the formation of plasma proteins. It seems obvious that protein or its derivatives, including amino acids, must be furnished the animal organism to achieve the production of new plasma protein. Certain protein already within the body as well as some of that received orally (or parenterally) may be commandeered for plasma protein formation. The exogenous supply will be given first consideration.

Food protein influences plasma protein production. Clinical observations substantiating this statement are numerous and have been reviewed by Moschcowitz (63). The hypoproteinemia and edema of malnutrition are cured by the ingestion of adequate food, provided that there are not also present any of the adverse factors—infection, liver disease (see below). Kerr, Hurwitz and Whipple (38) offered the first experimental evidence that diet influenced favorably the regeneration of depleted plasma proteins. In dogs *adequate* food protein has more recently been found to have a *qualitative* as well as a quantitative significance (65, 85). Per unit of protein fed, beef serum will favor the production of 3 times as much plasma protein as beef heart and more than 5 times as much as beef stomach. Let us examine the evidence upon which this statement is based.

If one places a dog upon a constant diet adequate in calories and other essentials but limited in protein to about 1 gram per kilo per day, the animal may gradually be depleted of plasma protein by daily plasmapheresis. If at first the dog is bled daily about one-fourth of its blood volume with reinjection of sufficient plasma-free red blood corpuscles suspended in a physiologic salt solution (plasmapheresis) to prevent anemia, a plasma protein concentration of 4 grams per 100 cc. will soon be reached. If the subsequent daily plasmaphereses are so adjusted as to maintain steadily this low plasma concentration, it will be found that smaller and smaller bleedings will suffice, but that a minimum will shortly be reached. This minimum, expressed as the grams of *plasma protein* removed from the dog during the course of one week,

may be called the *basal output*. This minimum quantity will remain constant for a given dog on a given basal diet for as long as the experiment lasts, and this period can cover at least a year (49).

If variations are introduced into the existence of this dog, a standardized biologic machine, their effect can be measured in a change in the quantity of plasma protein which must be removed from the animal in order to maintain standard conditions. Thus, if beef serum is added to the basal diet, a quantity of plasma protein will be produced in addition to the minimum basal output, equal to about 40 per cent of the protein in the beef serum fed (65). Such a response leads one to believe that the supra-basal, new-formed plasma protein comes (in whole or large part) from materials furnished in the added diet protein, and that the *basal diet protein* is wholly responsible for the *basal* output of plasma protein. In addition, variations in the quantities of plasma protein obtained from the feeding of different proteins justifies the assumption of qualitative differences.

Table 1 summarizes the proteins which have been tested in the formation of plasma protein. The tests represent the work of three different laboratories and as many different methods. The larger group of tests has been done in this laboratory by the method described above and the results are expressed as *grams of new plasma protein resulting from the feeding of 100 grams of the test protein.* When the test protein was the only protein in the diet, a "‡" follows the potency value; otherwise all test foods were added to a basal adequate protein intake. Thus, "liver, pork, raw, 17–33‡" indicates that when raw pork liver constituted the total protein of basal diets tested, the per cent return of plasma protein ranged from 17 to 33, in different dogs, not in the same dog. Reasons for such differences will be discussed below. Other differences will be noted. In one dog eating a basal ration containing the proteins of potato and bran flakes, added kidney protein was only one-fourth as well utilized for plasma protein formation as in another dog consuming a basal diet containing kidney. Conspicuous differences of this type are unusual and difficult of interpretation.

That plasma protein formation can be controlled by diet and that different proteins have different values in plasma formation are verified by an entirely different method (Weech and Goettsch, 84, 85, 83). These workers found that on a diet containing inadequate protein, furnished by carrots and polished rice, a decline in the serum albumin concentration occurred which was parallel to the decline in total serum protein concentration and also parallel to the plasma volume. If then

at the end of 3 weeks' decline a test protein were added to the diet, its effect on the serum albumin concentration could be measured during the fourth week. When this effect was determined for the same food on a number of different dogs, a rather wide range of biological response was found, but significant averages were established. For example, the

TABLE 1

*The potencies of various food proteins in forming blood plasma protein. Summary of all published data**

REFERENCE	HIGH POTENCY	MEDIUM POTENCY	LOW POTENCY
31, 65, 55, 47, 48, 49, 50, 91 (Rochester)	Serum, beef, 38† Serum, beef, dried, 28+	Salmon bread, 24†‡ Yeast, fresh, autoclaved, 23 Bran Flakes, 23 Kidney, pork, cooked, 22 Liver, pork, raw, 17-33‡ Kidney, pork, cooked, 19‡ Rice polishings, 19 Gizzard, 19 Thyroid, powdered, 19 Rice, polished, 19‡ Lactalbumin, 18 Skeletal muscle, beef, 18 Egg white, 17 Irish potato, powdered, 16 Salmon, 16‡ Liver, pork, raw or cooked, 15 Soy bean, 14, 8 Heart, beef, 13 Casein, 12 Liver extract, 12	Spleen, 10† Red blood cells, dog, 10 Brain, pork, 8 Stomach, beef, 7 Salmon, canned, 7 Gelatin, 2, 9 Pancreas, 6 Kidney, pork, cooked, 6 Zein, 0
83 (Columbia)	Serum, beef, 0.801	Egg white, 0.613 Beef chuck, 0.475 Beef liver, 0.436 Casein, 0.388	Gelatin, −0.093
59 (Yale)	Serum protein, beef, 0.63 Casein, 0.45 Lactalbumin, 0.38		

* See text for discussion.
† Figures mean grams of new plasma protein resulting from feeding of 100 grams test protein.
‡ Signifies potency as *basal* protein; all other figures indicate tests as *supplementary* protein.

assay values for beef serum in 11 different dogs ranged from 0.42 to 1.27, with an average value of 0.801; whereas the assay values for casein tested in 12 different dogs ranged from 0.07 to 0.58, with an average ("potency value") of 0.388, a statistically valid difference as pointed out by Weech. The potency values for other foods tested by this method are given in table 1 (Columbia). The biological variation

displayed by different animals under this test is of considerable interest. It may account for some of the sharp variations in assay values listed in table 1 from the writers' laboratory.

These potency values of Weech measure relative differences in *rate* of formation and not in total capacity for formation of albumin, as is measured for plasma proteins by the plasmapheresis technique. Evidently in the materials so far tested these two qualities are proportional, for the relative orders of potency determined by the two methods are similar. Such may not be true in all instances. It has been frequently noted that the addition to the basal diet of some proteins, for example soy bean, is followed by a much *prompter response in production of new plasma protein* than the addition of other equal or even superior materials, such as "gelatin + cystine + tyrosine" (table 2). The published data giving the *weekly totals* (55, 49) tend to obscure, though not entirely, this phenomenon so clearly observed in the daily determinations.

Melnick, Cowgill and Burack (59) assayed serum protein, casein, and lactalbumin and concluded that there was no significant difference in any of their potency values (see table 1). Their test procedure employed plasmapheresis and a basal protein-free diet (58). During the first week of this regime, removing daily 25 per cent of the blood volume of the dog, the circulating concentration of serum protein is reduced to 4 per cent or less. During the subsequent week of protein-free diet serum protein is removed as necessary to maintain the concentration near 4 per cent. In the third week, the test protein is added to the protein-free diet and in the fourth week the relation of serum protein removed to test protein intake is compared with the figures obtained for the second week and a potency ratio is calculated. For validity, their method rests on two assumptions. It is assumed first that of the total quantity of test protein fed, the fraction used for body nitrogen requirements other than serum protein formation can be determined, and this quantity is thought to be the minimum amount of the test protein which will keep the same dog in nitrogen equilibrium when it is in a normal non-hypoproteinemic state (57). Secondly, the reserve store of plasma protein building material of any dog is assumed to be measured as that quantity of plasma protein which can be removed in 6 bleedings during one week, each bleeding being equal to one-fourth the blood volume. As an important factor contributing to the formation of plasma proteins, the *reserve store* is discussed below. We believe the reserve store of plasma protein building materials cannot be removed

completely by this rapid procedure and that the assays recorded (59) are inaccurate because of the presence within the body of an unknown amount of protein reserve stores clearly evident in later experiments (56).

Do some foods favor albumin production? In 1932, Liu and co-workers (43) found animal protein twice as effective as vegetable protein in combatting edema and hypoproteinemia, but in a later publication, Liu and Chu (42) could find only slightly more favorable action in animal protein than in vegetable protein in raising the plasma protein concentration of two patients with nephrosis. As a matter of fact, neither type of protein was very definitely effective in this regard although a similar and considerable nitrogen retention and gain in body weight was obtained by each. The gradual rise in plasma protein concentration which occurred in both cases is probably better attributed to removal of incident infection than to dietary measures.

Animal experiments testing the relative capacities of animal and plant proteins in albumin and globulin formation present conflicting evidence (85, 55, 47). In long continued plasmapheresis experiments by which hypoproteinemia is maintained in dogs, the albumin:globulin ratio is almost invariably below normal. This reduction in the albumin: globulin ratio is found regardless of the kind of protein fed, although it may be greater with certain plant or grain proteins, ranging from 0.3 to 1.2. Only five different protein-containing foods of plant origin have been tested in such experiments (see table 1). These are soy bean, potato, bran, rice polishings, and rice. Soy bean is in a class by itself and is very promptly utilized and favors a high albumin:globulin ratio like many animal proteins. Only soy bean and rice have been fed unaccompanied by other protein and sample albumin:globulin ratios for the plasma protein produced are 1.14 for soy bean contrasted to 0.51 for rice (55). The soy bean was fed at an intake level of 1.9 grams protein per kilo body weight in a different dog. Such differences in intake may be of no significance as it has been observed that the albumin:globulin ratio during liver feeding is not appreciably altered by tripling the intake (dog 32-130 (65) compared with dog 34-152 (55)). Four dogs (65, 55) receiving protein from a potato-bran diet mixture had albumin:globulin ratios in basal periods ranging from 0.47 to 0.88. For comparison 4 other dogs (55, 47, 48, 49) receiving protein only from kidney or liver had basal albumin:globulin ratios ranging from 0.58 to 1.10. The lengths of these experiments and the intake levels of protein were comparable. It is evident that for albumin production,

sweeping conclusions concerning the group superiority of animal or of plant proteins cannot be safely drawn. However, certain plant proteins (rice and potato) in these experiments do favor a low albumin:globulin ratio or appear to favor the production of globulin when compared with certain standard animal proteins (meat, kidney and liver).

When a fasting dog is given plasma protein (whole dog plasma) by vein, the dog is kept in nitrogen equilibrium and even in weight equilibrium for many days (21). The dog uses up the introduced protein to supply its protein needs and as this goes on day by day the *albumin: globulin ratio remains unchanged* (66). This indicates that the body can and does use both albumin and globulin at about the same rate to carry on its normal internal protein metabolism. This would suggest that differences in the albumin:globulin ratio might be more frequently due to *variations of production* rather than to lack of use of the normal globulins.

Weech and Goettsch (84) find that in their method of assay, described above, the albumin and not the globulin fraction of the serum is influenced by the character of the diet. They do find an increase in the quantity of circulating globulin during the feeding of test proteins, but conclude that diet is not an important factor in influencing the formation of globulin.

Under the experimental circumstance of plasmapheresis, there are data which indicate that globulin formation is directly dependent on the diet (31, 65). For example, the same 100 grams of beef serum which produced 38 grams total plasma protein, produced approximately 21 grams albumin and 17 grams globulin (65). The addition of 100 grams bran flakes to a kidney basal diet (55) results in the formation of about 12 grams albumin and 11 grams globulin. Since it has been shown that 100 grams casein will yield only 5 grams albumin and 7 grams globulin, and 100 grams gelatin 5 grams or less of each, it becomes apparent that as measured by plasmapheresis *diet regulates globulin production equally as well as albumin formation.*

Amino acids. Since the experiments of O. Loewi in 1902 (44), demonstrating that the feeding of protein digests could maintain nitrogen equilibrium, much effort has been directed toward discovering the dietary essential protein constituents. For the growth of rats, these essential amino acids have been recently determined in the laboratory of W. C. Rose in a series of experiments announced by him in 1937 (69, 70). Rats fed a diet, the nitrogen of which is furnished by a mixture of pure amino acids, exhibit normal growth curves only when

appropriate quantities of the following ten amino acids are included in the mixture: threonine, valine, leucine, isoleucine, methionine, phenylalanine, tryptophane, arginine, histidine, and lysine. Moreover, Rose states that normal growth occurs if these amino acids are offered to the exclusion of all others. Such experiments invite speculation regarding the amino acid requirements for plasma protein synthesis. Since these ten amino acids support growth, they probably support the formation of new plasma protein in the growing blood volume of the young rat. Direct evidence of such a synthesis would be valuable.

In the dog by the plasmapheresis technique described above direct measurement of new formed protein can be made. Certain amino acids have been studied for their effect on the formation of new plasma protein in such standardized dogs (47, 48, 49, 50). When the addition of one or more amino acids to a basal diet is followed by an increase in plasma protein formation above basal it may be inferred that the added amino acids supplement the mélange available to the synthesizing mechanism (liver probably) from diet and body sources in such fashion as to permit the formation of more new protein. It seems probable that the added amino acids are at least in part incorporated in the new plasma protein and thus represent components essential for its synthesis in a depleted dog put to the strain of maximal regeneration of plasma protein. Whereas, positive experiments of this type are significant, it is obvious that a negative response does not imply that the added amino acid is unessential for plasma protein synthesis.

In table 2 are recorded the results of such amino acid feeding experiments. a, *Cystine*, with tryptophane or tyrosine, adds much plasma protein producing power to gelatin; b, *cystine*, with tryptophane and the other amino acids indicated, adds much plasma protein producing power to zein; c, *cystine*, with glycine, glutamic acid, and leucine adds much potency to the liver basal diet. Under certain conditions cystine qualifies as a *key amino acid* in plasma protein regeneration.

The last observation (c) raises a question as to the assumed participation of gelatin and zein in the first (a) and second (b) reactions. Calculated on the same basis (see footnote *, table 2), the potent amino acids with gelatin increase the basal output 121 to 191 per cent, with zein 108 and 134 per cent, and without either 107 to 121 per cent. Upon examining the original protocols (49, 50), however, the urinary nitrogen figures indicate a conservation of gelatin nitrogen of 40 to 70 per cent and of zein nitrogen of 90 per cent (of the added amino acid nitrogen of 100 per cent). It appears, therefore, that in these experiments the

TABLE 2

Summary of experiments on the influence of certain amino acids in plasma protein formation

	EFFICIENCY IN PLASMA PROTEIN FORMATION: PROTEIN OUTPUT PER CENT OF PROTEIN INTAKE			
Reference	(49, 50)	(49)	(48)	(47)
Dog number	36-196	37-6	33-11	34-152
Basal diets				
Liver	17	27	22	
Kidney				22
Supplements				
Gelatin + cystine + tyrosine + tryptophane	34			
Gelatin + cystine + tyrosine	39, 25			
Gelatin + cystine + tryptophane	32	41		
Gelatin + cystine	10			
Gelatin + tyrosine	5			
Gelatin + tryptophane	3±	3	33	
Gelatin + tyrosine + tryptophane	1			
Gelatin + cystine + phenylalanine	6	13		
Gelatin + methionine + tyrosine	12, 21			
Gelatin + methionine	5			
Zein + cystine + tryptophane + lysine + glycine + threonine	22			
Zein + cystine + tryptophane + lysine + glycine	28			
Tryptophane	0*		0*	
Lysine				0*
Histidine + lysine + arginine				26*
Cystine + glycine + glutamic acid				50*
Cystine + glycine + glutamic acid + leucine	107*			
Cystine + glycine + glutamic acid + leucine + isoleucine + arginine + lysine	117*			
Cystine + glycine + glutamic acid + leucine + tyrosine	121*			

* These figures represent the percentage increase in the basal plasma protein output induced by the amino acid supplement.

gelatin and the zein do participate effectively in the protein metabolism. It seems that the content of cystine, and probably leucine in liver limits the potency of the liver basal ration. Methionine deserves further

testing. Present indications are that methionine is not an efficient substitute for cystine in plasma protein formation, nor is phenylalanine for tyrosine. These experiments do not prove methionine or phenylalanine to be dispensable in plasma protein formation nor by their apparently negative reactions is such indicated for lysine, isoleucine, arginine, or threonine. In a preliminary report Rose and Rice (71) announce that nine amino acids are essential for maintenance of weight and nitrogen balance in the adult dog, the same nine amino acids which together with arginine Rose and co-workers found essential for normal growth in the rat (69, 70).

It appears reasonable to expect that in the near future a method will be perfected for the *parenteral administration* of the nitrogen requirements of the body including that for plasma protein formation. Its practical value in treatment of certain disease states is obvious. Observations by Elman (25) on dogs indicate that serum protein regeneration following severe hemorrhage may be aided by the intravenous administration of hydrolyzed casein with added tryptophane. Elman and Weiner (26) have carried out similar intravenous infusions on patients suffering from malnutrition and hypoproteinemia. They report that edema usually disappeared, serum protein concentration occurred, and nitrogen balance was easily achieved as a consequence of the casein digest injections. They found that these favorable results were not obtained when tryptophane and cystine (or methionine) were not added to the digest.

Miscellaneous exogenous materials tested in plasma protein regeneration. As would be expected in the normal animal, no substance has been found which will substitute for protein or amino acid in plasma protein synthesis. A recent report (68) indicating some utilization of *ammonia nitrogen* in body protein synthesis should prompt an early trial of such substances in plasma regeneration. Certain α keto and α hydroxy acids corresponding in other respects to essential amino acids have been found to substitute satisfactorily for such amino acids in the synthesis of body protein (70) and presumably the appropriate ones could be used in plasma protein formation. Parenteral *liver extract* and iron have both proved ineffective in stimulating regeneration in hypoproteinemic dogs (47).

Endogenous materials concerned in the formation of plasma proteins. *A reserve store of plasma protein building material is one subject for which the evidence is overwhelming and it may be accepted as a fact.* That such materials exist was first demonstrated by Morawitz

(62) in 1906, when he found that regeneration of the blood plasma proteins occurred during fasting following their acute depletion by bleeding. He combatted anemia by injection, simultaneous with the bleeding, of an equal quantity of red blood cells suspended in Locke's solution to which 3 per cent gum acacia had been added. This was the first use of a technique, later called *plasmaphaeresis* by Abel, Rowntree and Turner (1), which has proved valuable in studying the blood proteins. Morawitz found that by a large one-stage plasmapheresis the total plasma proteins of a dog could be reduced to about 2 per cent and that return to a normal level occurred during fasting, in 2 days in one instance and in 4 days or less in another. In the second instance, the dog was depleted again at 4 days, this time with reduction of the total plasma protein concentration to 3 per cent, and regeneration was observed, reaching a level of 5.4 per cent in 3 days, all in the fasting animal.

Kerr, Hurwitz and Whipple (37) and Smith, Belt and Whipple (76) added further evidence of a similar type to indicate that after rapid experimental depletion plasma protein regeneration can occur in the fasting dog. From such experiments it can be calculated that the quantity of materials available is at least sufficient to reform 40 to 60 per cent of the circulating mass of plasma protein originally present. By a similar approach but in a different species (rat), Cutting and Cutter (19) found that 40 per cent of the original mass of circulating protein could be regenerated within 12 hours after its removal. Seven days' fasting did not alter this remarkable response.

By another method in this laboratory, measurement of the *reserve store* of plasma protein building material has been made in many dogs (31, 65, 55, 47). The method and the calculation determining this store have been described in detail (48). The normal dog is depleted of circulating plasma protein by daily plasmapheresis while consuming a constant basal diet low but adequate in protein. With the normal dog it will be found necessary to remove more plasma (and plasma protein) in the initial days or weeks of the regime than in subsequent weeks in order to attain and keep a steady hypoproteinemia. This *excess quantity* of plasma protein removed in the first weeks of a prolonged period of plasmapheresis represents the *reserve store*. It is a greater quantity of protein than is represented by the difference in circulating mass of plasma protein before and during the experiment (i.e., (6 per cent − 4 per cent) × plasma volume = about 9 grams plasma protein in a 10 kgm. dog). Its measurement is graphically represented in figure A. The excess production noted in the initial 4 weeks (fig. A) must be

attributable to protein materials present in the body at the start of the experiment.

Probably largely influenced by the preceding nutritional history the quantity of *storage materials* available for plasma protein production has been found to vary rather widely from dog to dog. In actual experiments, the maximum quantities of reserve protein stores have usually not been measured since in the endeavor to attain basal conditions as rapidly as possible the animals have been *fasted* during the initial week or have been depleted by a previous period on a low protein diet.

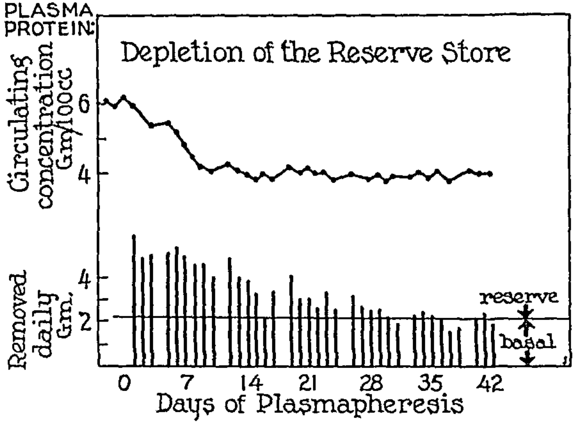

FIG. A

Thus the figures actually obtained are low. In 10 dogs, 10 to 14 kgm. in weight, the plasma protein production from reserve stores ranged from 24 to 68 grams (92). On other occasions in 2 of these dogs after both low protein diet and fasting, the reserves amounted to only 9 and 11 grams. Two larger dogs of 16 and 21 kgm. had reserves of 121 and 108 grams (30). It may be estimated that normal dogs have sufficient materials in storage to form a quantity of plasma protein one to two times that normally present in their circulations. This may amount to as much or more than the total protein content of the liver and indi-

cates that a large portion of it must be stored elsewhere. This amount is larger than that given by plasma protein regeneration during fasting (62, 37, 76) and probably more nearly represents what the animal can produce when the *only demand* on the stores is for plasma regeneration. Dogs once subjected to plasma depletion and thereafter allowed to return to normal exhibit *larger reserve stores* on subsequent depletion (48).

Other experiments reveal the process of *storage of plasma protein* forming materials. Such stores can be experimentally induced and reduced quantitatively. In a standardized protein depleted dog, plasmapheresis was discontinued for 2 weeks and the materials which ordinarily would have been removed as plasma protein were stored in the body depots, to be removed quantitatively as new plasma protein during the subsequent 3 weeks of plasmapheresis (48). During this period of storage, the plasma protein concentration rose to a maximum of 4.75 per cent and the quantity of stored material equaled 150 per cent of the entire mass of circulating plasma protein at the beginning of storage. Besides the evidence for storage, this experiment demonstrates the efficiency with which the body handles the exchange of protein materials—storage and removal from storage depots of protein material without loss of protein—"an ebb and flow" between plasma and body proteins.

A further example of the ability of the body to retain plasma protein forming materials in time of need is given by observations in a dog that ate over 400 grams of raw pork kidney in one brief meal (47). The dog conserved the nitrogenous constituents of this single meal as efficiently as it subsequently did a somewhat smaller quantity consumed over a period of 7 days. Plasma protein was formed from the nitrogen containing products conserved from this single meal for more than 14 days thereafter. This nitrogen "lag" or carry over is a well recognized though not clearly understood phenomenon which probably includes protein storage and the "ebb and flow" of proteins in the body as the terms are used in this review.

We have considered in the preceding paragraphs data bearing on the question of a body store of materials for *plasma protein synthesis*. Since these stores may be lessened by low protein diets and fasting, it is reasonable to suppose that they are a part of the general body stores measured by Addis, Poo and Lew (3, 5). The whole subject of protein storage has been reviewed by Cathcart (16) to 1921 and more recently by Luck (46).

Nature of the protein reserve store. A complete discussion of the nature of stored protein is beyond the scope of this paper. That the ni-

trogenous metabolites are stored as protein appears probable from the weight of evidence reviewed by Borsook and Keighley (13). These investigators present new data to indicate that in an adult man in nitrogen equilibrium and a urinary nitrogen excretion about 10 grams daily about half of this nitrogen comes from catabolism of stored protein, the extent of which storage is a function of the previous dietary history. This fraction of the total they term the "continuing" nitrogen metabolism. In this dynamic picture, extensive *synthetic processes* involving amino acids balance the *catabolic portion* of the "continuing" nitrogen metabolism. While the data are meager, the hypothesis is stimulating.

In the utilization of the reserve store for general nitrogen requirements or for plasma protein formation, it is characteristic that a large bulk of the store is used early and rapidly, a so-called "labile" portion. Three illustrations may be cited. 1. Addis, Poo and Lew (4) found that half of the total protein of the liver to be lost during a 7 day fast, is lost during the first 2 days. Considering that 40 per cent of the protein content of the liver of a rat is lost during a 7 day fast (5) liver storage protein appears readily available. Its rate of conversion is probably no greater, however, than that of the greater mass of stored protein found in the carcass of the rat. This mass lost 4 times as much protein as did the liver and from the data given it was available at the same rate, 50 per cent within the first 2 days. 2. Following rapid severe depletion of the plasma proteins in experiments discussed above (62, 76) the rate of regeneration from stored protein was most rapid in the first hours. 3. In the depletion of the reserve stores (48) the larger proportion is removed during the initial week or two but smaller quantities may continue to be contributed by them for as long as 5 or 6 weeks.

An explanation for the above phenomenon may involve the law of mass action and a tentative proposal may be advanced. When the steady state existing between body protein supply and demand is disturbed the rate of the reaction in the direction of a new equilibrium is determined by the product of the active masses of the substances reacting as well as by other constants and conditions (54). Thus, when the mass of stored protein is large, and change of diet, plasma depletion, body injury, or other disturbing factor is introduced, this mass will be converted at a faster rate than when the mass diminishes, other things being equal. Under such a concept the "labile" portion is merely that portion of the reserve store which is mobilized first and fastest and in other respects is not different from that made available more slowly.

Observations on the rate of mobilization of protein stores in response to acute demand for plasma protein indicate that the greater bulk of this reserve is not stored as plasma protein nor as material more easily converted into plasma protein than orally ingested protein. A small portion of the store sufficient to raise the concentration 0.5 per cent within 15 minutes after rapid acute plasma depletion (76) may be essentially preformed plasma protein. If a large portion of the store were readily available in only slightly modified form one would expect a rapid appearance of the plasma protein in the blood stream and a more prompt return to normal plasma protein concentration. Actually dietary protein (38) or intravenous amino acid mixtures (25) will accelerate the relatively slow regeneration of plasma protein from reserve stores.

A review of experiments measuring the reserve store of plasma protein forming materials (92) indicates that the store yields *albumin* on depletion in slightly more abundance than globulin, perhaps 10 per cent greater. This gives us a little insight into the potentialities of the reserve store.

Many investigators believe that to justify the term the *stored protein* should exist as a physically demonstrable entity comparable to glycogen and fat intracellular masses, and some claim to have found such protein deposits (8, 41). The careful chemical analysis of Luck (45) of the protein of the rat liver after storage had been induced, indicates all fractions of this protein to have participated equally in the storage process. Until more is known of the nature of protein as it exists within the cell, further discussion does not seem relevant.

Can plasma proteins be formed from essential (non-storage) body protein? The evidence so far speaks against this possibility, as it has been found that only 3 to 4 grams' plasma protein can be removed from a protein depleted dog during a week of fasting. This limited production is somewhat surprising as it is readily demonstrated that in experimental anemia a standard dog can produce large amounts (40 to 50 grams) of hemoglobin during fasting periods (20). It is probable that these anemic dogs do have some reserves of stored protein but the reaction is conspicuous and uniform and appears to involve conservation of protein-split products which contribute to the nitrogen fraction in the urine of the control dog (20).

The reserve store of protein may be defined as all protein which may be given up by an organ or tissue under uniform conditions without interfering with organ or body function. This definition indicates primary physiological significance, not anatomical. In view of the above

concept we may consider pertinent data. As noted above, dogs may be kept hypoproteinemic by plasmapheresis for months at a time and remain apparently normal in all other respects. *Such dogs are stripped of their protein stores and during periods of fasting or protein-free diet feeding can produce but little plasma protein.* What little plasma protein is removed may be attributed in part to a carry over from the preceding basal diet feeding or to plasma volume concentration or to uncontrollable variation in basal conditions. The quantity removed in one week during fasting ranges from 3 to 6 grams (65, 55) and during protein-free diet weekly periods averages 3 grams (48, 49). Even with the addition of the catabolism of an experimental *abscess* to a fasting, storage-depleted dog, only 4 grams plasma protein were removed in a carefully controlled experiment (50) corresponding to one previously reported (48) in an inadequately depleted dog. The presence of the abscess may introduce as a confusing factor an inhibition of the protein-forming mechanism. The abscess also makes available large quantities of protein split products, but apparently these are not used to form new plasma protein.

An observation by no means conclusive but of some interest in this consideration is one involving the catabolism of hemoglobin. When laked red blood cells were injected into the blood stream of a standard protein depleted dog, there resulted no production of new plasma protein which could be attributed to the injected material (49). This is an example of the failure of catabolism of an essential body protein to supply materials for plasma protein synthesis. Hemoglobin is admittedly an incomplete protein, among other deficiencies being low in cystine, and the lack of this amino acid may actually explain this reaction.

All in all we can give no adequate explanation why the plasma protein depleted dog cannot use effectively some of the waste products coming from protein wear and tear or disintegration. The dog is badly in need of new plasma protein and presumably the stimulus is maximal but the net output of this protein approximates zero when the food protein intake is zero.

Mechanism of plasma protein formation. The path leading to the formation of plasma proteins is obscure from the moment the amino acids enter the cell. *Amino acids* liberated by protein digestion are the ultimate source of the essential nitrogen and the preponderance of present opinion holds them to be the simplest units in the syntheses, although Alcock in a thought-provoking argument (6) questions this

assumption. Presumably given the proper mixture of amino acids the normal liver can form and release into the blood stream albumin, globulin and fibrinogen molecules. Bergmann (9) has contributed to our knowledge of the enzymatic processes which may be involved in this synthesis and further developments are eagerly awaited.

It is a truism that cells must form or synthesize protein if the body is to live. Any disturbance of this *mechanism* of protein formation is of great interest (10, 86) but conclusive evidence is hard to find. The fact that there can be an "ebb and flow" between plasma and cell proteins makes it difficult to produce evidence which is "leak proof."

Clinical evidence of long continued hypoproteinemia (77, 35) with demonstrable liver abnormality probably indicates an impaired protein forming mechanism. The Eck fistula liver with limited protein production under standard conditions (40) gives similar evidence favoring an impaired protein formation. There is also some experimental evidence that *infection* can limit the production of plasma protein in well standardized plasma depleted dogs (47). Presumably this means that the plasma protein forming mechanism is disturbed by the complicated body reaction to infection.

Prolonged hypoproteinemia per se causes no damage to the protein forming mechanism (56, 49). The lowered production of plasma protein noted in one report (47) was probably due to some deficiency in the diet not yet understood. Nor does any injury accompany the removal of plasma protein reserve stores by plasmapheresis. On the contrary, three different dogs subjected to long plasma depletion followed by a long rest period on kennel diet, subsequently in the next year when again subjected to plasma depletion showed much *larger reserve stores* of plasma protein building materials (compiled from 55, 47, 48, 49, 50). Moreover, the concentration of plasma protein has been found higher after recovery from long continued depletion than prior to it.

THE DISTRIBUTION OF BODY PROTEIN—A DYNAMIC EQUILIBRIUM. In evaluating the clinical and experimental data relating to plasma protein formation no knowledge would be more useful than an understanding of the processes involved in the distribution of protein within the body. The prevailing attitude appears to be that concentration of plasma protein is a direct measure of production and utilization. The tacit assumption has been that once formed unless obviously lost by hemorrhage, proteinuria, transudation, or inflammation, *plasma protein is static,* having only physico-chemical and immunological functions. We believe that recent evidence demonstrates plasma protein to have

another important rôle. It is a part of a *balanced system of body proteins.* A *steady state* or "ebb and flow" exists between it and a portion of the cell and tissue body protein. This portion called the *reserve store* has been described above. Since no better term has appeared than the one originally given to this steady state (32) we shall refer to it here as a *dynamic equilibrium.*

The evidence for this concept rests upon experiments which upset this dynamic equilibrium. We have noted above the circumstances under which protein materials may flow from cells and tissues into plasma. Experiments accelerating the flow from plasma into tissue are equally informative. By injection of dog plasma into protein-starved dogs, nitrogen equilibrium has been maintained for the periods of the experiments, 14 to 20 days (32, 66, 21), and probably could be maintained indefinitely. Obviously, such parenterally introduced protein is metabolized. Addis (2) has found it to increase the total protein content of the body. Of great interest are the observations by Howland and Hawkins (33) which demonstrate that the metabolism of the injected plasma protein does not result in an increase in blood amino acid concentration sufficient to be detected as increased urinary nitrogen and dextrose (phlorhizinized dogs). The control oral administration of plasma to a phlorhizinized dog was followed by the expected prompt and considerable increase in urinary nitrogen and sugar.

How are we to interpret such experiments? The injected plasma protein disappears from the circulation within 24 hours or less. The urinary nitrogen and sugar figures for 48 hours after injection of plasma compare with those obtained in the same dogs following injection of Locke's solution. It appears that the *metabolism of intracellularly ingested plasma protein* does not increase the flow of amino acids into the blood stream. Therefore, it appears probable that it does not increase the amino acid concentration of the tissues. But it can supply all of the nitrogen requirements of the body.

To return to the plasma-injected non-phlorizinized dogs, it is obvious that in nitrogen equilibrium they excrete more urea and ammonia nitrogen during injection periods than during periods of protein starvation. This increased nitrogen excretion must come either from catabolism of intracellularly ingested plasma protein or from increased catabolism of cell protein attendant upon the conversion of plasma protein to cell protein, or from both.

Speculation may be permitted as to the metabolic procedure of conversion of plasma protein to cell protein. It would appear simpler for

the body to split only partially and then realign the *large aggregates* into cell protein rather than be forced to digest such protein to its amino acid residues before being able to incorporate it into its individual cell protein (65, 33, 87). Some may hold that present concepts of specificity of protein structure are incompatible with synthesis from large aggregates (polypeptides or greater), but we do not find sufficient knowledge of the nature and synthesis of protein in vivo to rule out such a reasonable hypothesis.

On the other hand, it must be admitted that an alternative hypothesis is tenable, namely, that intracellularly ingested plasma protein may be catabolized to amino acids at the same rate as normal cell protein and then 1, deaminized, sparing the cell protein, or 2, formed into new cell protein while catabolism of the old continues. No present evidence would bar the simultaneous intracellular catabolism and anabolism of such proteins at rates consistent with the maintenance of the normal tissue concentrations (78, 79) of amino acids.

Perhaps further speculation may be tolerated. It is admitted that the body can be kept in nitrogen equilibrium by amino acids by mouth. There will be synthesis of plasma proteins in the liver in this type of experiment and these plasma proteins will be available all over the body to all cells. In an emergency (fasting) these same plasma proteins can supply all the nitrogen (and protein) requirements. Why must we assume that the complicated assembly of amino acids to the large protein aggregate is the task of each and every cell in the body? It would be possible for the liver, strategically situated as it is, to do the hard work of preliminary synthesis to spare this task to the body cells specialized for other purposes (e.g., muscle). These specialized cells under standard conditions could rely upon the reservoir of plasma proteins for their current needs. This thesis may not be accepted by many but at least it fits with some evidence which is accumulating.

One may in *conclusion* present the following as a concept of plasma protein metabolism and its relation to total protein metabolism. The ultimate source of construction materials is food protein furnishing amino acids absorbed from the intestinal tract and synthesized in the liver cells (and elsewhere) into plasma proteins. This same influx of amino acids, and/or the plasma proteins, support protein formation throughout the body. A part of the body protein forms a *reserve against adversity* in the sense that it can be circumspectly depleted without apparent injury to the body. The supplies of amino acids coming from outside the body and the demand for protein materials within the body

are in a constant state of balance with these protein reserve stores, a *dynamic equilibrium.*

If the body need for protein to build new cell protein, new plasma protein, or new hemoglobin is greater than the exogenous supply, this reserve store of protein will be drawn upon; if the body need is less, the store will be repleted. The maximum and minimum limits of these stores are an individual characteristic of each organ, tissue, and fluid and are determined by factors not yet understood.

When the body demands protein the plasma contributes its share and evidence from plasma injection experiments indicates that plasma protein molecules can be accepted as such by body cells and recast into specific cell protein without loss of nitrogen.

It seems to us probable that new plasma protein flows largely from the liver although some globulins may be formed elsewhere.

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THE STUDY OF INTERMEDIARY METABOLISM OF ANIMALS WITH THE AID OF ISOTOPES

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The study of intermediary metabolism is concerned with synthesis, interconversion and degradation of organic molecules. In adult animals in energy equilibrium the total amount, the distribution and the chemical constitution of body constituents are kept constant within narrow limits. The constituents of normal diets (proteins, fats, carbohydrates, etc.) are in principle the same as those which compose the animal body. The experimenter loses track of them as soon as they pass the intestinal wall and mix with the same substances in blood and organs. Very few experiments, therefore, on the intermediary metabolism of physiological compounds have been carried out with adult animals on normal diets, as in most instances the tracing of the food constituents was impossible. Our present knowledge of intermediary metabolism is based mainly, therefore, on experiments performed under such abnormal conditions as permit accumulation of intermediates or end-products: Use of unnatural compounds, or of diets unduly enriched or poor in test-substances, experiments on poisoned, or sick organisms or on isolated organs, tissues or extracts.

The isotopes of the elements C, H, N, S, O, which constitute organic matter provide tools to follow organic molecules directly in the intact and normal animal. There are two types of isotopes available for biological application: 1, the natural stable isotopes of hydrogen (1), oxygen (2), nitrogen (3, 4), carbon (5), and sulphur (4), all of which have been made available by Urey and collaborators through fractionation of the natural elements; 2, a great number of artificially produced radioactive isotopes. In the present review, which is concerned with the metabolism of organic compounds, only those radioactive isotopes will be discussed which have been used as markers in organic molecules; namely, ${}_{15}P^{31}$ and ${}_{16}S^{35}$, etc. No mention will be made of the extensive work in which radioactive isotopes have been employed in the study of the metabolism of ions.

Hevesy, who has recently reviewed his extensive work on radiophosphorus (6), was the first to realize the value of isotopes as biological tracers. As early as 1923 (7) when neither stable nor radioactive isotopes of biological elements were available, he followed the course of lead in animals by adding to it a minute amount of one of its natural radioactive isotopes (RaD). When heavy water became available, he carried out the first and one of the most fundamental experiments on water metabolism (8). He found the average time a water molecule spends in the human organism to be 14 days.

Most of the work on intermediary metabolism employing isotopes has been carried out with heavy hydrogen and heavy nitrogen. One paper by Tarver and Schmidt (9) has appeared in which radioactive sulphur was used for the study of the biological conversion of methionine into cystine. The biological introduction of radioactive iodine into the thyroid gland has been investigated by Hertz, Roberts and Evans (10). Hahn, Bale, Lawrence and Whipple (11) have used radioactive iron as a tool for the study of hemoglobin formation. They found inorganic iron to be poorly absorbed by normal, but readily by anemic dogs. Radioactive carbon has been employed for the study of photosynthesis (Ruben, Hassid and Kamen, 12). The short half life time of radioactive carbon makes its application to the study of intermediary metabolism of animals very difficult. Extensive work has been carried out with radioactive phosphorus. Phosphorus is not a true component of organic molecules but is found as phosphoric acid combined in ester or amide-linkage with organic molecules. The use of this isotope has proved of great value in the study of biological phosphorylation. In this review, phosphorus will be discussed only insofar as its use has direct bearing on the metabolism of organic molecules.

General properties of isotopes. Isotopes of the same elements have properties so similar that they are not appreciably fractionated in nature and the abundance is therefore practically the same everywhere. Even deuterium, which of all isotopes shows the greatest difference from its light analogue, is not measurably fractionated when organic compounds are formed in animals or plants. Dole (13) has shown in careful studies that the abundance of deuterium in honey, cholesterol, and benzene is the same as in water (see also Breusch and Hofer (14) and Steward and Halcomb (15)). The abundance of N^{15} in amino acids and proteins is practically the same as that in air (16). Living cells thus cannot distinguish between the isotopes of the same elements, but treat them practically alike.

Even if the deuterium content of water be artificially increased, the living organism does not fractionate it. Krogh and Ussing (17) after giving animals heavy water to drink, found the deuterium content to be the same in the water of all organs, blood and urine.

This inability of the cells to distinguish between isotopes also applies to isotopic organic compounds. A normal monoamino acid is a mixture of molecules, 99.63 per cent of which contain only N^{14}, and 0.37 per cent contain only N^{15}. The equal distribution of N^{15} in air and protein is proof that the cell treats both molecular species alike. If a physiological compound be administered in which the concentration of the isotopes is increased, this cannot constitute a material foreign to the body, as the living cell is accustomed to molecules containing "heavy atoms."

In work with deuterium, however, care must be taken not to raise its concentration too much. It has been shown by several workers (18) that high concentrations of heavy water (above 25 per cent) in the physiological fluids have a toxic influence, probably due to the changes in physical properties of the medium in which the biochemical reactions occur. As it has been shown that the rates of enzymatic reactions are different in heavy water (19) (either faster or slower), the biological effect of high concentrations may be due to disorganization of the delicately balanced rates. Concentrations of D_2O in the medium up to 10 per cent have no detectable influence. Organic compounds in which a large part or all of the hydrogen atoms has been replaced by deuterium may possibly be metabolized at different rates. Erlenmeyer *et al.* (20) have shown that succinic acid d_2 is dehydrogenated enzymatically at a slower rate than its normal analogue, and acetyl choline d_3 (21) has a smaller physiological action than ordinary acetyl choline. While the organic chemist may be interested in compounds with a definite and high deuterium content, the physiologist should employ substances with as low an isotope content as possible.

Stability and exchangeability of isotopes in organic linkage. When deuterium is employed as a marker of the carbon chain it has to be introduced into the compound in such a way that it will not be lost from the molecule either when dissolved in water or when transported in the aqueous fluids of the body. The deuterium atoms must be directly attached to carbon atoms. From our knowledge of organic reactions, it is in many cases possible to predict which hydrogen atoms will exchange their places with those of the aqueous medium. The use of deuterium has confirmed (22) the prediction that the hydrogen atoms in carboxyl, hydroxyl, amino, and other polar groups are ex-

tremely labile and exchange with the hydrogen of the aqueous medium at an immeasurably rapid rate. Such active hydrogen atoms are of no interest in metabolic research. Besides these extremely fast reactions, there exist others with slower rates: The hydrogen, attached to carbon atoms adjacent to ketonic groups, exchanges, due to enolization (23), and in some special cases (literature on exchange reactions, see 24, 25) other polar groupings may labilize the hydrogen of adjacent carbon atoms. Such slowly exchangeable hydrogen has been called semi-labile (26, 27). In most instances, however, the hydrogen of methyl and methylene groups is stable and is not removable by treating the compounds even at high temperatures with acid or alkali. Biological tracer experiments with deuterium are in general limited to such compounds as contain stably bound hydrogen atoms. As it is impossible on the basis of our present knowledge to predict with certainty whether the hydrogen atoms in a given compound will prove stable or labile, it is necessary in all metabolic experiments to test the stability of deuterium in the compounds administered as well as in those subsequently isolated from the living organism. Only in exceptional cases is it possible to use compounds with "semi-labile" deuterium, namely, when the rate of exchange is slower than that of the conversion of the substance into another with stable deuterium (28).

There is no indication that hydrogen atoms stable *in vitro* are labile *in vivo*. No enzymes have been observed which merely labilize carbon-bound hydrogen atoms without further involving the substance in a chemical reaction. The claim that proteolytic enzymes "exchange" stable hydrogen atoms in amino acids (29) has not been confirmed (30). The enzymatic replacement of deuterium by hydrogen due to dehydrogenases, *e.g.*, of deutero-succinic acid in ordinary water (20) COOH—CHD—CHD—COOH is not an enzymatic exchange reaction but a chemical reaction, involving reversible dehydrogenation and hydrogenation of the system fumaric $\rightleftharpoons$ succinic acid. If stably bound deuterium of organic compounds is removed, or if deuterium from the medium is introduced into stable positions of organic compounds, this reaction is always due to chemical processes (26, 30, 31, 32, 33, 34).

In contrast to hydrogen, carbon-bound nitrogen atoms do not exchange. The carbon-nitrogen linkage in all biological compounds investigated so far (35, 36) has proved to be stable. The same was found for the sulfur in sulfates (37) and in cystine (9). A biological replacement of normal N (or S) atoms by their isotopes can only be due to chemical reactions.

The oxygen isotope, O^{18}, while highly valuable as a tool for the in-

vestigation of chemical reactions (38) has not been used as a biological tracer for organic compounds. The high chemical "activity" of oxygen-containing groups and the exchangeability of carbonyl oxygen with the oxygen of water, restricts the application of this marker. Aten and Hevesy (39) have studied the excretion of sulfate ions, marked with heavy oxygen, and Day and Sheel (40) have followed the fate of heavy oxygen after inhalation by animals.

General methods for following metabolic reactions. *Procedure A.* The isotopic compound, prepared in the laboratory (or biologically) is administered to an animal. By determining the isotope content of organs or their constituents, it is in many cases possible to follow the transportation and degradation of the material fed. The isolation of highly purified substances may throw light on processes of interconversion of the carbon chain. If a deuterium-containing compound A is given and a compound B also containing deuterium is in turn isolated, the deuterium content in B may be taken as indication that B has been derived from A. This conclusion, however, is valid only if the deuterium content in the stable hydrogen of B is higher than that of the body fluids. The administration of deuterium compounds generally leads to the formation, due to their biological degradation, of some heavy water, which becomes equally distributed over all body fluids. In the course of chemical reactions (hydrogenations, condensations, etc.) small amounts of deuterium from this heavy water may in turn enter into organic molecules (see below). The concentration of deuterium introduced by such reactions, however, cannot be higher than that of the medium (body fluids) in which the reactions occur.

The nitrogen isotope is a marker of the nitrogenous group (amino, imino group, etc.) but not of the carbon chain. In work with amino acids and other nitrogenous compounds, the metabolic fate of the carbon chain and the amino group has to be considered separately. Both are known to follow different metabolic routes. If an amino acid is utilized in a biological reaction, either the carbon chain, or the amino group, or both, may be involved. It is therefore advantageous, for the study of nitrogenous compounds, to use two independent isotope markers, deuterium (or carbon if available) for the carbon chain as well as N^{15}. A complete study of sulfur-containing amino acids may require the use of even more independent labels in one substance.

Procedure B. The isotope is administered in inorganic form (hydrogen as heavy water, nitrogen as ammonia, phosphorus as phosphate, etc.). The presence of the isotope in organic linkage (deuterium in

stable positions) indicates the occurrence of chemical reactions. Heavy water as drinking water has been widely used for the study of the formation of fatty acids (26, 31, 41, 42), and cholesterol (26), as well as for the detection of chemical reactions in the amino acids of proteins (17, 33, 42, 43, 44). The method also has been used extensively by Bonhoeffer and collaborators (45) for the study of the formation of organic compounds in lower plants.

Only a few organic compounds have as yet been found which in the living animal do not take up stably bound deuterium from the heavy water present in the body fluids. The absence of deuterium can in many cases be taken as proof that the compound was neither formed nor involved in a chemical reaction affecting the carbon chain. Such substances must have entered the body as such with the diet and could not have been formed from other substances. The administration of heavy water is thus a convenient procedure to study "chemical inertia" of body constituents. Up to the present, only the fatty acids, linoleic and linolenic acid (46), and the amino acid, lysine (33), have been found to be inert in animals. Not all essential food constituents (for instance indispensable amino acids) show such inertia. It has been found (see below) that some of them are involved in continuous and reversible chemical reactions, such as deamination $\rightleftharpoons$ amination, a process which leads to the replacement of one stable hydrogen atom. Ammonia has been found to be utilized by animals to some extent (47, 48) and may thus be employed as a tool to follow amination processes as well as the formation of urinary urea. It has, furthermore, been shown to be a convenient tool for the study of protein metabolism in plants (49). Inorganic sulfates are utilized in the formation of organic sulfur compounds by plants (50) but not by animals (9, 51).

PREPARATION OF ISOTOPIC COMPOUNDS. The number of methods is too large to permit of detailed description and only those more general procedures will be mentioned which have been employed for compounds which have been used in biological research.

A. Deuterium compounds. 1. The simplest method is the hydrogenation of double bonds with deuterium gas. The method has been used for the preparation of the following compounds; propionic acid from acrylic acid (52), butyric acid from crotonic acid (52, 53), caproic acid from sorbic acid (52), stearic acid from linoleic acid (54), leucine from isopentenal diethylcetal and valine from the next lower homologue (55), homocystine and methionine from acetylene (56), ornithine from α-pyridone (57), and coprostanone from cholestenone (58).

2. Another procedure is based on the fact that some catalysts may under certain experimental conditions labilize carbon-bound hydrogen atoms. Many compounds when treated with hot concentrated D_2SO_4 exchange otherwise stable hydrogen atoms (59). A number of deuterium-containing fatty acids and amino acids have thus been prepared by this procedure (60, 61). The method introduces deuterium into fatty acids only at the α-carbon atom.

Another catalyst is active platinum (62, 63, 64, 65). A number of deutero-fatty acids, otherwise not easily obtainable, such as palmitic acid, lauric acid, capric, caprylic acid (61), and iso-caproic acid (66) have been prepared by heating the normal analogues in D_2O with platinum and a trace of alkali. The deuterium in these compounds seems to be equally distributed among the stable hydrogen atoms of the whole molecule.

3. An easy but wasteful procedure is the biological preparation. Animals, when given heavy water, or plants, when grown on heavy water, form a great number of deutero-substances. This method while theoretically unlimited is restricted in practice by the cost of heavy water. More economical is the biological conversion of one compound into another: deutero-oleic acid has been prepared by feeding mice with deutero-stearic acid (67).

4. Various other methods which might be applied also to the preparation of physiological substances have been reviewed by Erlenmeyer (68).

B. Nitrogenous compounds. The starting material is isotopic ammonia. The usual laboratory procedures for the synthesis were modified so as to furnish good yields calculated on the basis of the ammonia employed rather than on the carbon chain. In work with isotopic ammonia the value of the nitrogen is greater than even the most complex carbon chain. The common synthesis of amino acids by treating α-bromo acids with a large excess of ammonia is thus not practical.

Modifications of the phthalimide procedure of Gabriel (69) and of the catalytic reduction of α-keto acids in the presence of ammonia (Knoop and Oesterlin (70)) have proved of special value. The following compounds have been prepared: glycine, alanine, phenylalanine, tyrosine, norleucine, leucine, glutamic acid, aspartic acid (66), ornithine (57), α-amino-γ-phenylbutyric acid (34), sarcosine, creatine and creatinine (36).

Biological synthesis of N^{15} compounds is possible and occurred in all experiments when isotopic ammonia or isotopic amino acids were fed. The isotope content of the newly formed substances, however, is low.

C. Sulfur compounds. The synthesis of methionine from radioactive sulfur via benzyl mercaptan has been described by Tarver and Schmidt (9). Radioactive glutathione has been prepared biologically by growing yeast on a medium containing radioactive sulfate ions (50).

D. Phosphorus. Various radioactive phosphoric esters have been prepared, by Parnas (72) by means of enzymatic reactions, and one has been synthesized by laboratory methods by Chargaff (71).

PRINCIPLES OF ANALYSIS OF ISOTOPES IN ORGANIC COMPOUNDS. The analytical methods used for the tracing of biological compounds must be highly sensitive. The isotopic material administered in small quantities will mix with large amounts of the same (normal) material present in the animal, whereby the isotope is "diluted" by large amounts of the normal elements. The analysis of radioactive isotopes, while easier and more sensitive than that of stable isotopes, is not more accurate. The radioactivity may be measured by different devices, the most commonly used being the Geiger counter. The substance may be ashed or the activity of the organic material as such may be measured (73).

For deuterium analysis the compound is burned and the heavy water content of the water obtained is determined. The most common procedures employed are the determination of refractive index (74) or of density. Various procedures for density determination have been employed. The "falling drop" method (75, 76, 77) is, in the experience of the authors, the most practical; the submerged float method, while highly sensitive, is too laborious for routine purposes.

There are several inherent difficulties in the determination of deuterium by density or refractive index of water. Both methods are based on the measurement of second order differences which are highly sensitive to impurities. Furthermore, the methods are limited for routine purposes to samples of water of not less than 70 mgm. The measurement of heat-conductivity of hydrogen gas or water employed by Farkas and Farkas (78) and Bonhoeffer and collaborators (45) (see also 79) requires much smaller samples, but is useful only for high concentrations of deuterium. All these difficulties are overcome by the use of the mass spectrometer, which promises to become by far the most satisfactory method for the determination of deuterium in hydrogen.

Mass spectrometric analysis is the only practicable method for the precise determination of other stable isotopes in organic compounds. For the determination of N^{15}, the organic substance is subjected to the Kjeldahl procedure and gaseous nitrogen is obtained by treatment of

the resulting ammonium sulfate with alkaline hypobromite. The method requires about 1 mgm. of nitrogen. It is highly sensitive; the ratio of the abundance of the masses can be determined to within one per cent. The N^{15} concentration in normal nitrogen can thus be determined within 0.003 per cent (80). As the isotopic compounds administered to animals usually contain several atom per cent excess[1] of isotope. the analytical procedures used are good enough to trace the marker even when the compound is mixed with several hundred (or even a thousand) times the amount of its normal analogue.

Purification of isotopic compounds by "washing out" procedure. In biological work on conversion of an isotopic compound A into another one B, it is frequently necessary to prove that the isotope in B is not due to a contamination of B with A, which may not be detectable by other chemical or physical methods. Purification up to constant isotope content is not always sufficient as many impurities, especially those of homologues, are frequently not readily removable by crystallization or similar fractionating procedures. Proof that the isotope is actually present in B can be secured by the use of the "washing out" procedure, which is based on the fact that isotopic compounds cannot be separated from their normal analogues by ordinary laboratory procedures. To the crude isotopic compound B is added a large amount of normal nonisotopic A, and B is again separated from A by the usual procedures. B is still contaminated with the same proportion of A as before; the latter, however, has now the composition of the mixture of small amounts of isotopic A (which were present as impurity in B) and the large amounts of normal A added. The procedure does not remove A from B but replaces most of the contaminating isotopic A by normal A. The procedure was employed in work on the interconversion of fatty acids (67, 81, 82, 83), as the chemical separation of these compounds is almost always incomplete. It has also been employed in work with radioactive phosphorus (84) and in the purification of isotopic, optically active amino acids obtained from synthetic mixtures (85, 34).

THE METABOLISM OF FOOD AND BODY CONSTITUENTS. *A. Metab-*

[1] It is convenient to calculate the isotope concentrations in terms of "atom per cent excess": Glycine, containing 10 atom per cent N^{15} excess means that the nitrogen of the glycine molecules contains 10 per cent more N^{15} atoms than normal glycine, i.e., 10.37 per cent of all its nitrogen is N^{15}. The same terminology was used in work with deuterium: valeric acid with 10 atom per cent deuterium means that 10.02 per cent of all its hydrogen atoms are present in the form of D.

olism of fatty acids. The only isotope marker used thus far for fatty acids is deuterium. All natural fatty acids contain stable hydrogen atoms in $-CH_3$, $-CH_2$ or $-CH$ groups. In the early work on transportation of fats in the animal organism, linseed oil was used that had been partially reduced with deuterium gas. Such a deutero-fat represents an ill defined mixture of fatty acids, some of which may even belong to the elaidic series and should be employed with caution in experiments on intermediary fat metabolism. It is preferable to employ isotopic fatty acids of known structure.

1. *Transport and deposition of dietary fatty acids.* Animals given marked fatty acids rapidly and extensively deposit them in the fat tissues and in the fat of the internal organs (81, 86, 87, 88). The highest concentration is always found in the internal organs (86), particularly in the liver (Barret, Best and Ridout (88); Cavanagh and Raper (87)) indicating a high "activity" of fat metabolism in this organ. The isotopic fatty acids are found not only in the neutral fat but in the phospholipids (87). Rapid deposition was also found after administration of individual isotopic fatty acids (stearic acid (82), oleic acid (67), palmitic acid (83)). The phenomenon, however, is restricted to higher fatty acids and is not observed when lower acids, such as butyric and caproic acids, are administered (52). These are rapidly and completely degraded. When given in large quantities to animals with ketosis, deutero-butyric acid is partly excreted as deutero-β-hydroxybutyric acid in the urine (Morehouse (53)).

The extensive deposition of the higher fatty acids is observed even when the animals are held at constant weight. The proportion of dietary fat deposited seems to be almost independent of the amount administered. Even when the diet is low in fat (1 per cent), a considerable part is deposited. In all such experiments in which the animals do not gain weight, the fatty acids utilized for energy requirements must be a mixture of dietary and body fatty acids. As much as 45–50 per cent of the dietary fatty acid administered in such small quantities was recovered from the body (83, 86).

As fatty acids are present in animals not in the free form but in ester linkage, the presence of isotopic dietary acids in the body fats indicates replacement of tissue acids involving continuous formation of ester linkages in normal animals. There are two reactions possible which might lead to fatty acid replacement: 1, continuous breakdown into glycerol and fatty acids and resynthesis of the total fat molecule, or 2, continuous ester shifts. Both reactions are known from *in vitro*

experiments to be induced by lipase. The isotope method determines only end-results and cannot yet distinguish between the two reactions. Both reactions may occur *in vivo*.

2. *The source of liver fats.* Barret, Best and Ridout (88) have made a careful study of the source of liver fat under different experimental conditions. By feeding, in a preliminary period, deuterium-containing fats to mice, they "marked" the fat tissues of the animals by the deposition of dietary fats. When anterior pituitary extracts or carbon tetrachloride were then given, the deuterium content in the liver fats clearly indicated their origin from the depot fat. However, the fat accumulating in the liver on diets low in protein and lipotropic factors (choline) had a low deuterium content. The fat accumulated in the liver under these conditions was thus not derived from the depot but probably synthesized in the liver from dietary carbohydrates. The authors found no transfer of depot fat to the liver when the animals were placed on a protein-rich diet. On the latter diet, the deuterium content of the fat depots is reported to remain constant for considerable periods, an indication of the stability *in vivo* of deuterium present in fatty acids.

3. *Interconversions of fatty acids.* While the ability of animals to form fatty acids from carbohydrates or proteins had been definitely established, proof for interconversions of fatty acids was lacking. The classical balance experimentation was unable to decide whether a fatty acid newly formed was derived from another fatty acid or from a non-fatty substance. The deuterium technique has proved to be a valuable tool for the study of such processes. It has not only established the ability of animals to convert one fatty acid into another, but has demonstrated that such reactions occur continuously even when the total amount and properties of the body fats do not change.

a. Desaturation and saturation of fatty acids. After administration of deuterium-containing saturated fatty acids to mice, the unsaturated fraction contained deuterium indicating its origin from the saturated acid (84). Not only is oleic acid continuously formed from stearic acid, but palmitoleic acid, which is a normal constituent of rat fat (89), is continuously formed from palmitic acid (83). Linoleic and linolenic acid, however, are not formed by desaturation (see later).

The reverse process, saturation of unsaturated acids, has also been demonstrated in mice (67): the animals received deuterium-containing oleic acid prepared biologically. The stearic acid of these animals contained deuterium, indicating its origin from the deutero-oleic acid.

b. Shortening of fatty acid chain by two carbon atoms. Mice degrade deutero-stearic acid to palmitic acid (82). The degradation of palmitic acid to lower acids (lauric and myristic acids) was followed in rats (83). The reactions occur continuously in normal animals. The findings may be taken as a new support for the theory of Knoop and Dakin of one-sided β-oxidation of natural fatty acids in normal animals.

c. Elongation of fatty acids by two carbon atoms. The feeding of small amounts of deutero-palmitic acid to normal rats results in the formation of deutero-stearic acid (83). This establishes continuous occurrence of *direct* elongation of the chain of palmitic acid by two carbon atoms.

All these conversions observed must have occurred without first breaking down the fatty acids into smaller units, as intermediate disruption would have led to a loss of most of the deuterium from the molecule.

Aliphatic alcohols as intermediates in fatty acid metabolism. Cetyl alcohol ($C_{16}H_{34}O$) as well as octadecyl alcohol ($C_{18}H_{38}O$) had been found to be minor but normal constituents of feces (90). They are not products of bacterial reduction, but are continuously secreted into the intestinal lumen (91). The origin of the aliphatic alcohols has now been traced to the fatty acids (83): after the ingestion of small amounts of deutero-palmitic acid, the fecal alcohols contained a high concentration of deuterium. On the other hand, animals rapidly convert these alcohols into the corresponding fatty acids: Feeding deutero-cetyl alcohol results in the formation of deutero-stearic acid as well as deutero-palmitic acid; deutero-octadecyl alcohol gives rise to deutero-palmitic acid as well as deutero-stearic acid. These processes are so rapid that the amounts of deutero-acids deposited in the depot fats of the animals are practically the same whether the deuterium is administered in the form of acid or of alcohol. As all these reactions have been studied in normal animals on their normal diets, they must be normal events. The alcohols may represent intermediates in the process of elongation of carbon chains described above.

Synthesis of fatty acids from small molecular units. No experiments have yet been carried out by following directly the formation of fatty acids from isotopic sugars or amino acids. It is questionable whether deuterium can be employed for such experiments. The intermediates of sugar metabolism are compounds of low molecular weight, of which most or all of the hydrogen is exchangeable. Deuterium originally introduced into the molecules of sugars or amino acids would be trans-

ferred from the carbon chain of the compounds to the aqueous medium at the instant when such labile intermediates are formed. In the second step, the condensation of smaller molecules to form larger ones, only normal hydrogen from the body fluids would be introduced. The loss of deuterium in the course of such reactions was observed with deutero-propionic acid. This substance is known to form a corresponding amount of glucose in diabetic animals. Administration of deutero-propionic acid to a diabetic dog resulted in extra production of glucose, which did not contain deuterium (52).

The introduction into organic compounds of hydrogen from the aqueous medium in the course of condensations or during hydrogenations can be used as a general procedure to study chemical reactions in normal animals (reaction B, p. 220). If fatty acids are formed from sugars or amino acids in a medium of heavy water instead of ordinary water, large amounts of deuterium must enter the reaction and take up stable positions in the newly formed fatty acid molecules. In the course of direct interconversions of fatty acids described above, only small amounts of deuterium will enter the molecule, as all these reactions are limited to a small number of carbon atoms. The formation of fats from sugar or amino acids must lead to a fixation of much more deuterium. When mice (or rats) are given heavy water to drink, a continuous formation of deuterium-containing fatty acids has been observed (26, 31, 41, 42). The process goes on until the deuterium concentration (in atom per cent) in the saturated fatty acids is half of that in the medium (body fluids) in which they were formed (26, 46). On extending the time of the experiment, no further rise in deuterium concentration occurs. The fatty acids must thus be formed by a process by which at least 1 out of 2 hydrogen atoms in the fatty acids is derived from the water of the medium, while the other normal atoms must have been derived from the starting material. Only a synthesis obtained from smaller units, such as the intermediates of carbohydrate or protein metabolism can be responsible for this result.

All theories of fat formation from sugars formulate aldol condensations and Cannizzaro reactions as intermediary steps. Bonhoeffer and collaborators (92, 93) have shown that these two reactions, when carried out in a medium of heavy water, do not lead to an introduction of stably bound deuterium into the resulting compounds, even though water enters the Cannizzaro reaction. This might explain why only half of the hydrogen atoms in the newly formed fatty acids are derived from the water of the body fluids. They are probably introduced in the course of reduction.

The administration of heavy water can thus be used as a means to follow synthesis of fatty acids in animals. The process has been studied in adult mice of constant weight on a fat-free diet. The observed new formation of fatty acid must have been coupled with a simultaneous degradation of an equivalent amount of fatty acids, *i.e.*, the uptake of deuterium measures the molecular regeneration[2] of fatty acid molecules in normal animals.

It is clear that such reactions are dependent upon the general conditions under which the animals are kept. However, the experiments are reproducible. It was found that in adult mice, half of their fatty acids is regenerated in 5 to 9 days,[3] *i.e.*, in a period much shorter than had been generally anticipated.

The rate of uptake of deuterium into different fatty acid fractions and individual fatty acids has been studied. The saturated fatty acids, stearic acid, as well as palmitic acid, were found to be regenerated at approximately the same rate (26, 46). The regeneration of unsaturated acids proceeds at the same rate as that of the saturated acids; however, the total concentration of deuterium in the unsaturated fraction is lower than that of the saturated one. This is due to the fact that the highly unsaturated fatty acids are not regenerated.

The biological inertia of linoleic and linolenic acids. In contrast to all the other fatty acids investigated so far, linoleic and linolenic acids do not "take up" deuterium from the heavy water of the body fluids (46). This finding must be taken as proof that they were not formed either directly from small molecules nor from other fatty acids, such as stearic or oleic acid. These highly unsaturated acids must have been derived directly from the diet. This finding is in agreement with the results of earlier authors (95) who found these acids to be indispensable dietary constituents.

[2] Up to the present we have employed the term "turnover" for the process of simultaneous synthesis and destruction. We now prefer the word *regeneration*. "Turnover" implies the replacement of molecules by the same kind from any source, also by those of the diet. All biological substances, even inorganic ions are "turned over," but only certain organic compounds are regenerated, i.e., resynthesized after destruction. Hevesy in his work on isotopic phosphorus has used the word rejuvenation for the replacement of only part of a molecule, e.g., the replacement of phosphate in phospholipid or phosphocreatine by other phosphorus.

[3] It is a mistake to determine the time necessary for *complete* replacement of molecules by newly formed ones, i.e., the time necessary for *total regeneration*. As such reactions follow exponential curves, their time is infinite.

The metabolism of phospholipids. The most abundant phospholipids, lecithin and cephalin, are compounds of glycerol, fatty acids, phosphoric acid, and a base (choline or aminoethanol). Isotopes maybe used to study the metabolism of any of these components. While in a few cases, the uptake of deutero-fatty acids into phospholipids was studied (87), most of the work has been carried out with radioactive phosphorus. The first experiments with this tool were performed by Chievitz and Hevesy (96) and by Artom and collaborators (97, 98). Hevesy (6) has recently reviewed his extensive and important work.

Hahn, Hevesy and Lundsgaard (99) found by administration of radioactive phosphate that the average time an atom of phosphorus remains in the body of a rabbit is about 30 days. When radioactive phosphates are administered to animals, the phosphorus of the phospholipids displays radioactivity (6). Phosphorus in these compounds is linked to two alcohol groups, namely, that of glycerol and that of choline or cholamine. The appearance of new phosphorus can only be due to chemical reactions, not to mere exchange. It indicates either complete or partial breakdown followed by resynthesis of the phospholipids. As the reaction has been observed in normal animals, even when no fatty acids were given, phospholipids, like fats and fatty acids, must be continuously regenerated. Hevesy and Lundsgaard (100) fed fat and radioactive phosphorus to a dog in order to determine where the increased lecithin was synthesized. They conclude that the site of formation is not the intestinal tract. In another experiment, Hevesy and Hahn (10) injected biologically prepared radioactive phosphatide into the blood stream of a rabbit and found that one-half of it disappeared from the blood in 1.5 hours. After a lapse of three hours, one-third of the phosphatides was in the liver. Robinson and collaborators (102) showed that tissue slices of liver, kidney and intestine can incorporate inorganic phosphate into phospholipids. In the intact animal different organs have a different turnover of phospholipids. Artom, Sarzana and Segrè (98), Perlman, Ruben and Chaikoff (103), and Fries, Ruben, Perlman and Chaikoff (104) (see also 84, 105), have investigated the rate of turnover of phospholipids in various organs of rats. These authors find that the phospholipids of the liver show the highest activity, the small intestine and kidneys slightly lower, while the lowest activity is found in the brain (106). Artom, Sarzana and Segrè (98) have, on the basis of reasonable assumptions, investigated mathematically the rates of the replacement of

labeled phosphorus in phospholipids. Jones, Chaikoff and Lawrence (107) have studied the regeneration of phospholipids in tumors. The rate in this tissue varies but is roughly the same as that of the liver. Perlman and Chaikoff (108) found the addition of choline and betaine accelerates phospholipid metabolism in the liver, while cholesterol retards it. Lecithin and cephalin are regenerated at about the same rate (Chargaff (109)).

Hevesy and Hahn (110) have given radioactive phosphate to laying hens. They found the administered phosphorus in the yolk, albumen and shell of the egg. They conclude that the bulk of the phospholipids in the egg is synthesized in the liver and transported through the plasma to the ovaries. The corpuscles play no part in this transport. Extensive phospholipid formation was observed in the chick embryo during development but none in the yolk (6). Labeled phospholipid appears in the egg yolk within six hours after administration of phosphate (111).

Steroid metabolism. The regeneration of cholesterol has been studied in mice on a cholesterol-free diet (26) by giving the animals heavy water to drink. The process is slower than with fatty acids, the half time being about 15–25 days (as compared to 5–9 days for fatty acids in the same animals). The amount of deuterium (in atom per cent) introduced into cholesterol is the same as has been found for fatty acids; during cholesterol synthesis at least every second hydrogen atom is derived from the aqueous medium. This indicates that cholesterol is synthesized by condensation of small molecular units. No appreciable sterol regeneration was observed in the developing chicken egg.

The process of coprosterol formation in dogs and humans has been studied with deuterium (58, 28). Deutero-cholestenone and deutero-coprostanone, which reappear as deutero-coprosterol, are both probably biological intermediates. Cholic acid isolated from bile after feeding deutero-coprostanone (112) did not contain deuterium. The ketone is probably not a precursor of bile acids.

CARBOHYDRATE METABOLISM. As mentioned earlier, no experiments have been carried out by administration of isotopic sugars. One paper on the uptake of deuterium from the body fluids into newly-formed liver glycogen has been published by Ussing (113). The amount of stably bound deuterium in the glycogen of rats fed with sugar and heavy water was much higher than could be accounted for by a process

by which glucose molecules are merely coupled with each other. It was suggested that the monohexose was first split into smaller units.

A number of experiments on the formation of sugars in lower plants, as well as on alcohol formation by yeast, have been published by Bonhoeffer and collaborators (45).

PROTEIN METABOLISM. The proteins are the most complex compounds of the cell, containing a very large number of functional groupings. Most metabolic work at present is essentially a study of the reactions of smaller units (amino acids) and their linkages. From the standpoint of metabolism, each amino acid has at least two parts, the carbon chain and the amino group. It is desirable to employ for the study of their metabolism two different isotopes, one for the carbon chain and one for the amino group. Isotopic nitrogen has been available for such studies for only three years (3). The investigation of protein metabolism with N^{15} is still in the first stages of development.

The use of deuterium as a marker for the carbon chain of amino acids is somewhat more restricted than was the case with fatty acids and cholesterol. Some of the carbon-bound hydrogen in a few amino acids is "semi-labile," *i.e.*, slowly exchangeable at elevated temperature (29, 27, 44, 17, 45). The exchange at high temperature is probably the result of such reactions as partial racemization, equilibrium processes (*e.g.*, glutamic acid $\rightleftharpoons$ pyrolidone carboxylic acid) and of the mobility of the hydrogen atoms ortho to the phenolic group in tyrosine (27). Deuterium present in such positions may be lost partly or even completely during hydrolysis of proteins. Amino acids marked with deuterium at such positions may be employed with the greatest care only. Except for glycine, in which all the carbon-bound hydrogen atoms are "semi-labile," all amino acids investigated so far contain some stable hydrogen atoms (not exchangeable even under the drastic conditions of protein hydrolysis), which, when replaced by deuterium, may be utilized as markers for the carbon chain.

The fate of dietary nitrogen. The nitrogen excreted by adult animals kept in energy and nitrogen equilibrium has generally been considered to represent mainly dietary nitrogen, together with a small amount originating from repair of "wear and tear." This theory of exogenous and endogenous nitrogen metabolism has been tested by adding small amounts of amino acids containing N^{15} to the ordinary stock diet of adult animals to constant weight. Up to the present three amino acids have been tested (dl-tyrosine (114), l(−)-leucine (85) and glycine (115).

In all experiments only small proportions of the isotopic nitrogen

were recovered in the excreta and in the non-protein nitrogen fraction, about half was found to be introduced into the body proteins. This distribution seems to be typical and even when the isotopic nitrogen was given as ammonium salt, a considerable part of the nitrogen was recovered from tissue proteins (47, 48).

As all animals maintained constant weight and total protein content, the introduction of dietary nitrogen must have been due to chemical reactions of the proteins which led neither to an increase of total protein nor to a change of its structure. Isotopic nitrogen in amino acids or ammonia thus provides a tool of value not only for tracing the fate of the particular compound given but for the study of chemical reactions of the body proteins.

Direct replacement of amino acids in proteins by dietary amino acids. Part of the new nitrogen in the body proteins is due to direct introduction of the marked dietary amino acid, *i.e.*, of the original carbon chain with the amino group still attached to it. The investigation of this reaction requires the presence of an isotope marker in the carbon chain. Ussing (116) fed a rat, previously fasted, with a protein hydrolysate into which stable deuterium had been introduced by racemization in D_2O at 170°. Most of the deuterium was, therefore, located at the α-carbon atom of the amino acids. The deuterium content of the proteins of the liver of the animal indicated that at least 10 per cent of the liver protein and 2.5 per cent of the muscle protein were "newly" formed in the course of three days.

This biological reaction has been more thoroughly investigated with l(−)-leucine containing deuterium attached to the carbon chain as well as N^{15} (85). After this compound had been fed to normal rats for three days, leucine was isolated from liver proteins and from the proteins of the rest of the body. Both preparations contained the two markers, indicating that carbon chain and amino group were introduced together. From the deuterium analysis it was calculated that at least 32 per cent of the carbon chain of the dietary leucine had been introduced as leucine into the proteins of the animals.

The corresponding values obtained when tyrosine or glycine were administered were similar but not as definite, as the data were calculated on the basis of isotopic nitrogen only. As will be seen later, the nitrogen becomes too easily detached to be of much value for tracing the carbon chain. The same applies to the deuterium atom attached to the α-carbon atom, which is removed simultaneously with the detachment of the amino group.

The replacement of amino acids in the protein by identical amino

acids of dietary origin requires two successive processes, the first of which must involve the opening, the second the closing, of at least two peptide linkages.

Continuous deamination and amination of the amino acids in proteins. Only a fraction of the new (dietary) nitrogen introduced into the proteins of animals is present in the form of the amino acid to which it was originally attached. Amino acids other than that administered contain considerable quantities of isotopic nitrogen. This finding is based on the isolation and isotope analysis of pure amino acid samples from the proteins of liver, intestinal wall or total carcass of animals given dl-tyrosine (114), l(−)-leucine (85), glycine (115) or ammonia (47, 48). The following amino acids were thereby found to have taken up nitrogen from others: glycine, tyrosine, leucine, glutamic acid, aspartic acid, proline, histidine, arginine. None was ever found in lysine.

As the nitrogen-carbon linkage is stable (35), the presence of isotopic nitrogen in the other amino acids must be due to chemical reactions involving both the compound that "yielded," and that which "accepted" the marked nitrogen. Two processes are conceivable which may be responsible for the result: 1, continuous destruction and resynthesis of total molecules, or 2, continuous deamination and reamination of amino acids. Reaction 1 may be responsible for the introduction of new nitrogen into dispensable amino acids but not into some indispensable compounds (the carbon skeleton of which the animal organism is unable to synthesize).

More detailed investigation of leucine (85) and histidine (117), both of which are indispensable, showed that they had accepted nitrogen from other amino acids. This must have been due to mere amino shift, without synthesis of the carbon chain. l(−)-Leucine containing the two isotopes was found to have yielded isotopic nitrogen and had accepted normal nitrogen without equivalent destruction of the deuterium-containing carbon chain. Histidine, isolated from animals given isotopic ammonia, was degraded in the laboratory to β-imidazolelactic acid. The isotope was found in the α-amino group but not in the ring. Deamination and amination had occurred, not synthesis.

The amino shift is responsible for the fixation in proteins of the bulk of the dietary nitrogen. The direct introduction of amino acids previously described plays only a minor rôle. In the 3-day experiment with leucine only 30 per cent of the new nitrogen in the protein was still attached to leucine, while the remaining 70 per cent was found in other

protein constituents. The corresponding values for dl-tyrosine were 25 per cent in tyrosine and 75 per cent in amino acids other than tyrosine.

The isotope technique thus reveals the occurrence of rapid amino shifts among the amino acids of proteins in animals. A number of biological reactions had already been formulated for free amino acids, and these might have been the cause of the results. All of them require the preliminary deamination of at least one of the reacting members to the corresponding keto acid. Reductive amination of such keto acids in the presence of ammonia liberated from other amino acids has been proposed by Knoop (118). Von Euler and collaborators (119) have demonstrated this enzymatic reaction to occur *in vitro* with ammonia and keto-glutaric acid. The enzyme is specific for glutamic acid. According to Braunstein and Kritzmann (120) and other workers, glutamic and aspartic acids can, by a process of direct "transamination," transfer their nitrogen to other α-keto acids, to form new amino acids. This process was found to be reversible, *i.e.*, keto-glutaric acid may form glutamic acid from other amino acids which are simultaneously converted into the corresponding α-keto acids. On the basis of these recent findings, glutamic and aspartic acids should play a central rôle in the utilization of ammonia (primarily involving fixation of nitrogen at the α-amino group) and in the transmission of nitrogen from the carbon chain of one amino acid to others.

The results obtained with N^{15} strongly support the theory that the dicarboxylic acids play a central rôle in protein metabolism. Whenever isotopic amino acids (85, 114, 115) or ammonia were given (47, 48), the isotope concentration in the dicarboxylic acids was much higher than in any other amino acid (except the one in which the isotope was administered). The concentration of isotope in glutamic acid was always somewhat higher than in aspartic acid.

The α-amino nitrogen is linked in the peptide to two carbon atoms. The process of amino shift, like that of direct introduction, requires the opening, at least temporarily, of peptide linkages for the liberation of the amino groups of the reacting amino acids.

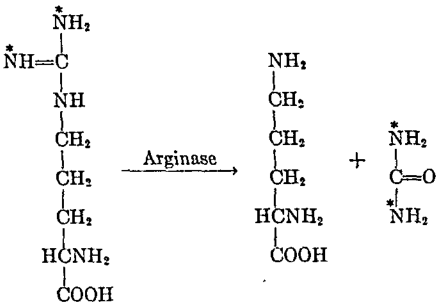
The "relative activity" of proteins of various organs and body fluids. As the total amount of proteins did not increase in the experimental animals, the fixation of dietary nitrogen must be due to continuous chemical processes which lead to no final quantitative or qualitative changes. The concentration of new nitrogen can be taken as a measure of relative "chemical activity" of the organ proteins.

Various organs have a different share in the "uptake" of dietary nitrogen. The serum protein always has the highest concentration of isotopic nitrogen, in the viscera (liver, intestinal wall, kidney, spleen, heart, testes, etc.) it is somewhat lower, while in muscle and skin it is lowest.

Although the concentration of "new nitrogen" in muscle and skin is very low, these two organs, by reason of their large size, play the most important rôle in the total replacement process. They usually contain more than 80 per cent of the total "new nitrogen" retained by the total proteins of the animals.

Deuterium may also be used to study chemical reactions in body proteins. Stekol and Hamill (43), also Krogh and Ussing (17, 42, 44), gave rats heavy water to drink and found stable deuterium in the body proteins. The latter authors found that electrical stimulation of muscles accelerates the introduction of deuterium. Stekol and Hamill hydrolyzed the proteins and found both tyrosine and cystine to contain stably bound deuterium. Foster, Rittenberg and Schoenheimer (33) repeated the experiments with two series of mice and isolated glycine, leucine, tyrosine, proline, arginine, histidine, glutamic acid, aspartic acid, cystine and lysine. All of these amino acids, with the exception of lysine, contained considerable amounts of stably bound deuterium. The authors concluded that this must be the result of chemical reactions, in which even indispensable amino acids (except lysine) take part and suggested the occurrence of continuous deamination and amination. Later experiments with N^{15} proved this suggestion to be true. It is of interest that in the experiments with deuterium, as well as with N^{15}, glutamic acid displayed the highest activity.

Activity of amidine group in arginine. The administration of isotopic amino acids or ammonia invariably results in the formation of isotopic arginine in the body proteins (47, 48, 85, 114, 115). Arginine has four nitrogen atoms, two in the ornithine moiety and two in the amidine group ($NH = \underset{|}{C} - NH_2$). The degradation of the biologically formed isotopic arginine to urea (or ammonia) and ornithine showed that most of the isotope was present in the amidine group. Only in some cases was a small amount detected in the ornithine. According to the theory of urea formation through the ornithine cycle, proposed by Krebs and Henseleit (121), arginine is an intermediate in urea formation and the amidine group in arginine may be considered as "potential urea." The findings with N^{15} support this theory: Arginine is



The asterisks designate nitrogen atoms marked with isotope.

continuously degraded into urea and ornithine and the latter is reconverted into arginine. In the second stage of the process, isotopic nitrogen enters the amidine group. Arginase, however, supposed to be one of the enzymes involved in the process, reacts only slowly (122) or not at all with arginine in peptide or protein linkage. It is highly probable that the arginine isolated from the proteins was involved in urea formation during the frequent intervals when it was in the free state.

Continuous formation of arginine from ornithine and the replacement of protein-arginine by such newly-formed arginine have been directly demonstrated by employing deutero-ornithine, the feeding of which to adult mice of constant weight resulted in the presence of large amounts of deutero-arginine in the proteins of the animals (57).

The biological inertia of lysine. In contrast to all other amino acids hitherto investigated, lysine does not take up deuterium from the body fluids (33) nor N^{15} from other nitrogenous compounds (47, 85, 114).[4] Lysine may be degraded in the animal, but it is not involved in any other reactions affecting its carbon chain, such as synthesis or reversible amination. This finding is of interest in connection with the results of earlier workers who found that lysine, in contrast to many other indispensable amino acids, cannot be replaced in the diet of growing animals by the corresponding α-hydroxy acids (123), nor by its optical

[4] The amino acids investigated are: glycine, tyrosine, glutamic acid, aspartic acid, proline, cystine, histidine, arginine (also its ornithine moiety) and leucine.

antipode (124). As soon as more data on the chemical "activity" or inertia of amino acids become available, it may be possible to classify them on the basis of the chemical "activity" of their amino groups.

Methionine and cystine. Tarver and Schmidt (9) have synthesized dl-methionine, the sulfur of which was radioactive. After feeding this compound to rats cystine isolated from the proteins was radioactive. The experiment proves that the sulfur of methionine is utilized for cystine formation.

Glutathione and hippuric acid formation. Waelsch and Rittenberg (125) fed rats isotopic glycine, and isolated glutathione as the copper compound from the liver and other organs. The material contained a high isotope concentration, indicating a rapid regeneration of the glutathione. It is probable that most of the isotope in this peptide was present in the glycine moiety. The isotope content of the latter must then have been much higher than that of the glycine in the proteins of the corresponding organs, which was also isolated and analyzed. The simultaneous administration of benzoic acid, resulting in the excretion of isotopic hippuric acid, did not appreciably influence the isotope concentration in the glutathione. The experiments indicate that the replacement process in glutathione is much faster than in the proteins of the organs from which it had been isolated.

It had earlier been shown (126) that the administration to rats of isotopic glycine together with benzoic acid results in the excretion of hippuric acid, only part of which contains the isotopic tracer. A large amount of glycine in the hippuric acid must thus have been supplied from other sources, probably from proteins. The result may be taken as an indication that large amounts of glycine in proteins are available for detoxication and are so utilized, even when an abundant supply of "extra" glycine is administered.

Biological inversion of amino acids of "unnatural" steric configuration. The ability of animals to invert some amino acids of "unnatural" steric configuration into their "natural" isomers has been definitely established. Du Vigneaud and Irish (130) demonstrated that phenylaminobutyric acid, whether given as the "natural" or "unnatural" enantiomorph, is excreted in the urine as the acetyl product of the l form, the configuration of which corresponds to that of natural amino acids. Du Vigneaud and collaborators (34) have now followed this process, *i.e.*, inversion and acetylation with the aid of both N^{15} and deuterium. When the l-("natural") form with N^{15} in the amino group was given, the acetylated product in the urine contained almost the maximum amount of N^{15}, whereas the same substance isolated after the feed-

ing of the "unnatural" form contained only very little isotope. During inversion most of the nitrogen of the amino group must thus have been removed and replaced by nitrogen from other sources. When the reaction was studied with rats in which the body fluids contained heavy water, the phenylaminobutyric acid isolated from the urine contained one atom of stable deuterium which, by degradation of the amino acid, was proved to be attached to the α-carbon atom. This introduction of new hydrogen into the α-position was observed whether the "natural" or "unnatural" amino acid was administered. The results seem to indicate that acetylation of amino acids is connected with dehydrogenation of the amino acid, a reaction previously suggested by du Vigneaud and Irish (130). The experiment supports the view that biological inversion of an "unnatural" amino acid involves almost complete deamination to the corresponding keto-acid, followed by asymmetric amination.

THE METABOLISM OF CREATINE AND CREATININE. Most of the creatine in animals is present as phosphocreatine. Hevesy and Rebbe (94) have shown that the administration of marked phosphate results in a rapid formation of radioactive phosphocreatine in muscles of frogs. Three hours after injection, 78 per cent of the original phosphoric acid was replaced. The phosphocreatine thus shows a rapid regeneration. The same result is found when small amounts of creatine containing N^{15} are given to normal adult rats (36). Most of the isotope is then found in the creatine of muscles and internal organs, where it must have replaced creatine linked to phosphoric acid. The findings are in agreement with the concept that phosphocreatine is rapidly turned over in the course of muscle work.

The administration of isotopic creatine results in the excretion of isotopic creatinine in the urine, definitely establishing the conversion of creatine into creatinine (36). If no creatine is administered with the diet, body creatine is the only source of urinary creatinine. This result was obtained in experiments with rats which were given isotopic creatine in a preliminary period. As the result of the replacement of muscle and organ creatine by the isotopic dietary material, the animals then contained "marked" body creatine. In the subsequent periods, when the animals received a creatine-free diet, the urinary creatinine had the same isotope content as the body creatine.

The conversion of creatine into creatinine, reversible *in vitro*, is irreversible *in vivo*. When isotopic creatinine was administered, it was not converted into isotopic creatine but excreted unchanged.

By determining the rate of disappearance of isotopic creatine from

the muscles (by isotope analysis of urinary creatinine) in normal adult animals on a creatine-free diet, *i.e.*, when the total body creatine is constant, one measures the rate of regeneration of the creatine molecule proper. In contrast to all amino acids and most fatty acids, the creatine molecule was found to be only slowly regenerated: 2 per cent of the body creatine of adult animals was newly formed per day, which is about the same amount as is excreted in the urine as creatinine. There was no evidence in these experiments of any degradation of the creatine molecule: neither urinary ammonia nor urea contained isotope.

PHOSPHORYLATION IN MUSCLE. Radioactive phosphorus has proved to be a valuable tool for the study of such reactions. As stated above, the phosphate in phosphocreatine is replaced rapidly by inorganic phosphate (94) and the same was found for 2 of the 3 phosphates in adenosinetriphosphate by Meyerhof and collaborators (127). That the remaining phosphate is replaced very slowly was shown by Korzybski and Parnas (128), who injected radioactive phosphate into pigeons and rabbits and then isolated inosinic acid showing only slight radioactivity.

Some of the transfer reactions of phosphorus are direct and proceed without the intermediation of inorganic phosphate. Cozymase does not take up inorganic phosphate when involved in transfer. Even during the intramolecular rearrangement 3-phosphoglyceric acid $\rightleftharpoons$ 2-phosphoglyceric acid, inorganic phosphate does not enter the reaction (127). Similar results were obtained by Hevesy, Parnas and collaborators (129) who studied the formation of adenyltriphosphate from adenylic acid in the presence of isotopic phosphoglyceric acid: The adenyltriphosphate displayed a much higher radioactivity than the inorganic phosphate. The latter could thus not have been an intermediate.

ISOTOPIC COMPOUNDS AS INDICATORS FOR CHEMICAL REACTIONS OF BODY CONSTITUENTS. The results obtained by feeding isotopic physiological compounds to animals in energy and nitrogen equilibrium, *i.e.*, to animals of constant body composition, can scarcely be reconciled with the classical concept of independent exogenous and endogenous varieties of metabolism. The experiments with isotopic phosphates, fatty acids and amino acids have all shown that the body constituents are involved in continuous chemical processes and that there exists a close interaction between the food materials and the body components. Peptide, ester, and probably other linkages of the complex body ma-

terials, open and close continually. The amino acids, fatty acids or other units temporarily liberated, mix with others of the same species of whatever source, diet or tissue. By this mixing process they become indistinguishable as to their origin. While in the free state, the organic units take part in a variety of chemical reactions of which only few have as yet been investigated with isotopes; the amino acids are deaminated and may transfer their nitrogen to other deaminated molecules; fatty acids are interconverted into other types, and molecules newly derived from entirely different sources (*e.g.*, fatty acids from carbohydrates) enter this pool of liberated molecules. Parts of this metabolic mixture reënter vacant spaces in larger molecules (amino acids into proteins, fatty acids into fats, etc.). Phosphate ions continuously liberated from phospholipids and other organic phosphorus compounds mix with the relatively large amounts of phosphate ions in the body fluids and part of this mixture reënters the organic substance in the second stage of the process. As stated above, it is not at present possible to decide with certainty whether liberation and reintroduction of smaller units is associated with complete or only partial breakdown and resynthesis of larger molecules (fats, proteins). The findings of Bergmann (131) on enzymatic replacement of amino acids in peptides suggest that the observations with isotopes *in vivo* are also the result of replacement processes.

The nitrogen excreted in the urine may be regarded as part of the metabolic pool originating from the interaction of dietary nitrogen with the relatively large quantities of reactive body nitrogen, and the same applies to other atoms or molecular groupings in dietary and tissue substances (carbon dioxide in expiration, phosphates in urine, etc.). The rapid interaction of many substances makes the isotopic compound an indicator not only for the particular material administered, but for the large number of other molecules with which the compound interacts.

The fact that the living organism, in contrast to the dead material, keeps constant the form of the cells and organs as well as the chemical structure of the large molecules has led many investigators to believe that the tissue enzymes, which show their destructive power during autolysis, lie dormant during life and are "activated" only when their function is required. The results obtained with isotopes make such a supposition unnecessary. The experiments indicate that all reactions, for which specific enzymes and substrates exist in the animal, are carried out continually. The isotope method, therefore, in contrast to

others, does not require special experimental conditions for the investigation of metabolic processes. The normal adult animal on its normal diet is the best experimental subject.

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RELATION OF NICOTINIC ACID TO PELLAGRA

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Not infrequently a single observation may unite two apparently unrelated fields of scientific endeavor. The resulting relationship often increases the rate of progress in both fields. An excellent example of such mutual assistance is the recent progress in the fields of respiratory enzymes and vitamins. The work on cocarboxylase was given a definite stimulus when it was found to be related to vitamin B_1, the chemistry of which had taken years to elucidate. A similar relationship exists between the vitamin, riboflavin, and the yellow respiratory enzyme. Studies on the constitution of pyridine coenzymes undoubtedly aided in the identification of the antipellagra factor.

It is the purpose of this review to summarize briefly both the nutrition work and the enzyme work which led to the recognition of nicotinic acid as the antipellagra factor and then to discuss the significance of this relationship.

Pellagra was first recognized in Spain two centuries ago by Casal. Stannus and Gibson (1934) have reviewed the early observations on this disease. In 1830 the census in northern Italy revealed that in many areas 5 per cent of the population were suffering from pellagra. The disease was also endemic throughout France from 1818 to 1880, but after the latter date the disease is said to have practically disappeared. A large incidence has also been reported in the Balkan States. The disease is considered rather rare in Germany, Holland, Belgium and Great Britain.

During the past fifty years pellagra has been a definite problem in the United States. The first cases were recognized in 1863 by Gray in New York and Tyler in Massachusetts. A few sporadic cases were reported until 1907 when several were discovered in the mental asylums of South Carolina and Alabama. Voegtlin (1919–20) has reviewed the early studies on pellagra in the United States. Up to 1912, 30,000 cases with a fatality rate of 40 per cent had occurred. Clinical studies were started in Savannah, Georgia, shortly thereafter and the Thompson-McFadden Commission began its work in South Carolina in 1912.

Voegtlin postulated in 1914 that there was a causal relation between a mainly vegetable diet and pellagra and that a restricted vegetable diet might be defective on account of 1, a deficiency or absence of certain vitamins; 2, the presence of some toxic substance, and 3, a deficiency of certain essential amino acids. Goldberger, Waring, and Willets (1915) were the first to show conclusively that pellagra can be prevented by means of an appropriate diet. Three southern institutions were selected in which a large number of cases of pellagra had occurred for several years. In the fall of 1914 the diets were supplemented with milk, fresh meat, eggs, and dried beans. One year after the change only one out of 244 pellagrins had a recurrence of the disease whereas on the basis of previous experience a recurrence of about 50 per cent was expected. Goldberger and Wheeler (1915) produced typical pellagra in prison volunteers by placing them on a restricted diet.

It is beyond the scope of this review to describe in detail the symptoms of pellagra. The early symptoms are weakness, lassitude, anorexia and indigestion, followed by sore and ulcerated mouth and diarrhea. The typical dermatitis usually makes the diagnosis easy. According to Smith and Ruffin (1937) the patient may present in addition to the typical dermatitis on the exposed surfaces, seborrhea over the face and neck, hyperkeratosis over the bony prominences of the body, and lesions about the genitalia. Nervous manifestations occur in many cases of pellagra. Lorenz (1916) concluded that the psychosis accompanying pellagra has the characteristics of toxic psychoses in 90 per cent of the uncomplicated cases. The relation of sunlight to clinical manifestations of pellagra has been thoroughly reviewed by Smith and Ruffin (1937). The high incidence of pellagra during May to July is explained on the basis that there is a gradual accumulating deficiency during the winter months and when the patients become exposed to increasing intensity of the sunlight the acute symptoms of pellagra appear.

Sebrell (1938) has recently discussed the diet in the treatment of pellagra and points out that in the mild cases careful feeding is all that is necessary, but emphasizes that the severe cases require more energetic treatment. Spies (1935), however, demonstrated that the mortality rate among patients with severe pellagra, the majority of whom were addicted to alcohol, could be reduced from 54 per cent to 6 per cent in a series of 125 cases. McLester (1934) maintained that the death rate from severe endemic pellagra remained high irrespective of the method

of treatment. Later work by Spies, Chinn and McLester (1937) showed that endemic pellagra, like so-called alcoholic pellagra, responds to the administration of a high caloric, high protein diet, large amounts of yeast, and adequate rest.

The first report on the value of liver extract in pellagra was that of Ramsdell and Magness (1933) who found rapid clinical improvement in two cases upon the daily injection of 2 cc. of extract. The importance of liver extract was not accepted by all workers but the evidence (reviewed by Sebrell, 1938) was soon sufficient to warrant its use in severe cases of pellagra. The finding that liver extract had anti-pellagra activity gave considerable impetus to further animal work because liver extract had also been found to be effective in black tongue by Goldberger and Sebrell (1930).

Voegtlin (1919–20) described his early experiments with animals in which it was demonstrated that it was possible to produce in animals on a restricted vegetable diet both histological and chemical changes which in all respects except skin lesions were identical with those found in pellagra. Later Chittenden and Underhill (1917) found that dogs fed on a diet of crackers made from milled wheat flour, boiled peas, and vegetable fat developed diarrhea, loss of appetite and ulceration of the oral mucous membranes. The symptoms promptly disappeared when fresh meat was supplied. Wheeler, Goldberger, and Blacklock (1922) concluded that the condition was identical with canine black tongue, a disease in dogs so named because of the necrotic areas on the tongue; and later Goldberger and Wheeler (1928) were able to produce black tongue in dogs on diets similar to those eaten by humans in pellagrous areas. Goldberger and associates (reviewed by Sebrell, 1938) continued to collect evidence that substances capable of curing black tongue in dogs were equally effective in human therapy and vice versa. The two diseases were considered to be analogous and the term pellagra-preventive or black tongue-preventive factor was applied to the factor originally postulated by Goldberger and Tanner (1925). Since the activity in yeast was stable to autoclaving, the factor was associated with the more heat stable fraction of the B complex. However, Underhill and Mendel (1928) found that the condition produced in their dogs was cured by carotene and carotene-rich substances. This discrepancy has now been explained by Smith, Persons and Harvey (1937) on the basis that the oral lesions in both the Goldberger and Underhill types of canine black tongue are the results of infection with the fusospirochetal group of organisms secondary to lowered tissue resistance.

McCollum, Simmonds and Parsons (1919) tested the diet used by Goldberger and Wheeler (1915) on growing rats and found that the rats were able to live for many months but showed no increase in weight. Goldberger and Lillie (1926) produced a pellagra-like condition in rats on a diet deficient in the B complex but supplemented with an alcoholic extract of corn. They concluded that although it was highly probable that the pellagra-like condition in the rat was the analogue of pellagra in man, additional evidence was necessary to establish the relationship beyond doubt. The difficulties encountered in producing a deficiency of the antipellagra factor in rats is now well known and some of the problems encountered in attempting to use the rat for assay of this factor have been reviewed by Sebrell (1938) and Elvehjem (1938).

Guha (1931) found that liver extract 343 produced good growth in rats on a vitamin B_2 deficient diet and Salmon and Guerrant (1931) showed that liver extract contained four times as much vitamin G (B_2) as a sample of brewer's yeast. We now know that the growth obtained on liver extract in the case of rats was not due to the antipellagra factor but to other members of the B complex. However, the fractionation work on liver extract which followed not only aided in the identification of the antipellagra factor but other factors as well. In this connection it is interesting to note that the studies of Voegtlin, Neill and Hunter (1920) on the prophylactic value of foods in the treatment of pellagrins showed that the administration of yeast and rice extracts over a considerable period of time in large amounts failed to modify the course of the disease, with the possible exception of one case, but the administration of liver preparations was followed by a definite improvement.

Due to the difficulties encountered in the production of pellagra-like lesions in rats, Elvehjem and Koehn (1935) used chicks for assay of their concentrates. Chicks placed on a heated grain ration develop a typical dermatitis which is readily cured by low levels of liver extract. Although it is now established that the activity of liver extract in this syndrome is not due to nicotinic acid but rather pantothenic acid (Woolley, Waisman and Elvehjem, 1939; and Jukes, 1939), the work with chicks did give a new approach to the problem. It was soon found that the factor active for the chick was separate from riboflavin, which Kuhn, György, and Wagner-Jauregg (1933) had just isolated and shown to have growth-promoting properties in rats. Concentrates rich in riboflavin were completely inactive for the chick while purified fractions

from liver retained their potency after removal of riboflavin. Lepkovsky and Jukes (1935) also found riboflavin inactive and introduced the name "filtrate factor" for the active substance in the filtrate after removal of the riboflavin with fuller's earth.

Koehn and Elvehjem (1936) repeated the work they had done with chicks using dogs, since they recognized that there was no evidence to show that the pellagra-like condition obtained in chicks was identical with human pellagra. Black tongue was produced in dogs on a modified Goldberger diet. Concentrates of riboflavin from liver extract were found to be completely inactive in the prevention or cure of black tongue, but fractions from which the riboflavin had been removed and which were shown to be potent for chicks were highly active. Birch, György and Harris (1935) also found that the addition of riboflavin had no curative action on dogs that had developed black tongue on Goldberger's diet. Sebrell, Hunt and Onstott (1937) obtained similar results through the use of more carefully controlled conditions and relatively large doses of pure riboflavin. The complete inactivity of riboflavin in the treatment of human pellagra has been reported by Dann (1936) and Fouts, Lepkovsky, Helmer and Jukes (1936). Previous to the above studies Rhoads and Miller (1935) reported that it was impossible to produce black tongue in dogs on a diet low in vitamin G (as measured by rats) and suspected that black tongue was due to the lack of some other factor than vitamin G.

Further purification of the liver fractions gave concentrates which contained very small amounts of solid matter and showed high activity in both chicks and dogs and finally Elvehjem, Madden, Woolley and Strong (1937) demonstrated the activity of nicotinic acid in the cure of black tongue and isolated nicotinic acid amide from the concentrates. The activity of nicotinic acid in the treatment of black tongue was soon verified by Street and Cowgill (1937), Dann (1937), Sebrell and coworkers (1938), and Ruffin, Margolis, Margolis, Smith and Smith (1938). When the antipellagra factor became available in pure form, it was possible to clearly differentiate it from the chick antidermatitis factor. Fouts, Helmer, Lepkovsky, and Jukes (1937), Dann (1937), and Mickelsen, Waisman and Elvehjem (1938) have shown that nicotinic acid or the amide are completely inactive in the prevention of chick dermatitis.

It is interesting, now that the chick factor and the antipellagra factor have been differentiated, to look back upon some of the difficulties encountered. In their early work Lepkovsky and Jukes (1935) were inclined to believe that the material in the fuller's earth adsorbate was

the antipellagra factor because Goldberger, Wheeler, Lillie and Rogers (1928) had found the antipellagra activity in a fuller's earth adsorbate of an aqueous extract of yeast. Later Fouts, Lepkovsky, Helmer and Jukes (1936) cured pellagra with liver filtrate from which riboflavin and vitamin B_6 had been removed by adsorption on fuller's earth. Nicotinamide is not readily adsorbed on fuller's earth and the activity obtained by Goldberger and co-workers must have been due to the adsorption of nicotinamide along with other inert material. Jukes (1937) differentiated the chick factor and the antipellagra factor on the basis of the differences between the distribution of the two factors in foods. An important difference was in the case of wheat germ which was low in the chick factor and supposedly high in the antipellagra factor. Yet in more recent studies wheat germ has also been found to be low in nicotinic acid. Koehn and Elvehjem were fairly well convinced that the chick factor and the antipellagra factor were identical until the actual isolation of nicotinic acid. It is easy to see how this was possible when the chemical properties of the two compounds are compared. A preparation of pantothenic acid free of nicotinic acid is very hard to prepare.

The recognition of nicotinic acid as the antipellagra factor did not result without some help from the earlier studies on nicotinic acid. Huber (1867) first prepared nicotinic acid in 1867 by the oxidation of nicotine. Its isolation from biological material was not achieved until 45 years later when Funk (1913) isolated nicotinic acid in crystalline form from yeast concentrates which possessed antineuritic activity. The acid itself, however, displayed no activity in curing pigeon beriberi. At about the same time Suzuki, Shimamura and Odake (1912) isolated nicotinic acid from rice polishings. Williams (1917) impressed by the common occurrence of nicotinic acid with the antineuritic vitamin in several natural substances again tried nicotinic acid, trigonelline, as well as other pyridine derivatives for antineuritic potency, but none of them caused any permanent improvement in polyneuritic fowl. Trigonelline, the methyl betaine of nicotinic acid, was isolated from plant material by Jahns (1885). Ackerman (1912) found that dogs given fairly large amounts of nicotinic acid excreted in the urine about equal amounts of trigonelline and nicotinuric acid (the dipeptide of nicotinic acid and glycine).

Except for the work of Szymanska and Funk (1926), who attributed an appetite-stimulating and weight-preserving action to nicotinic acid

and the amide, very little interest was shown in the possible rôle of pyridine derivatives in living systems until the work of Warburg and von Euler in 1935. Warburg and Christian (1935) characterized nicotinic acid amide as one of the hydrolysis products from the coenzyme which they had isolated from blood and which is now known as coenzyme II. Kuhn and Vetter (1935) isolated nicotinic acid amide from heart muscle and von Euler, Albers and Schlenk (1935) from cozymase.

This work gave new impetus to the application of these compounds in the field of nutrition. Von Euler and Malmberg (1936), using a diet similar to the Sherman-Bourquin diet supplemented with thiamin and riboflavin, found no growth response with the acid or the amide although the rats receiving the acid lived longer. Funk and Funk (1937) found larger food intake and better growth in rats and pigeons on certain rations when given the acid and especially the amide. Frost and Elvehjem (1937) observed a growth stimulus from nicotinic acid when fed with adenylic acid to rats on a factor W deficient diet. However, none of these responses was of sufficient magnitude to attribute to nicotinic acid real vitamin-like properties.

The significance of nicotinic acid in the nutrition of microörganisms was also recognized at about this time. Knight (1937) showed that nicotinic acid was an essential growth factor for Staphylococcus aureus and that nicotinic acid was present in most active preparations of the Staphylococcus growth factor. Mueller (1937) showed that nicotinic acid is essential for diphtheria bacillus; Koser, Dorfman and Saunders (1938) for dysentery bacillus; and Fildes (1938) for Proteus.

The activity of nicotinic acid in the treatment of black tongue suggested its therapeutic use in human pellagra and the first report of its successful use was made by Spies, Cooper and Blankenhorn, and by Fouts before the Central Society for Clinical Research in Chicago, November 5, 1937. Published description of the work followed in rapid order (Fouts, Helmer, Lepkovsky and Jukes, 1937; Harris, 1937; Smith, Ruffin and Smith, 1937; Spies, Cooper and Blankenhorn, 1938; France, Bates, Barker and Matthews, 1938; Sydenstricker, 1938; and Rachmilewitz and Glueck, 1938). At first only a few cases were treated but later papers (Spies, Grant, Stone and McLester, 1938; and Sydenstricker, Schmidt, Fulton, New and Geeslin, 1938) reported its use in hundreds of cases. Several workers (France, Bates, Bailey and Matthews, 1938; Spies, Bean and Stone, 1938; Frontali and Ferrari, 1938; Bogart, 1938;

and Spies, Aring Gelperin and Bean, 1938) have emphasized that the most dramatic response of a pellagrin to nicotinic acid therapy is the disappearance of the acute mental symptoms.

Spies, Bean and Ashe (1939) make the following summary concerning its use:

> In cases of acute or chronic pellagra in relapse it will: (a) cause fading of the fiery red lesions of the mucous membranes and diminish the Vincent's infection associated with it, (b) in most cases, restore to normal disturbed gastrointestinal function, (c) restore to normal the mental function deranged moderately or severely in acute pellagra, (d) cause fading of the dermal erythema but not cure chronic changes of the skin. In cases of subclinical pellagra, the vague ill-defined symptoms disappear and in persons subject to recurrence of the disease the development of clinical pellagra is prevented. In both clinical and subclinical pellagra, the sense of well-being, one of the attributes of health, is restored.

Spies, Walker and Woods (1939) have shown that infants and children may also suffer from nicotinic acid deficiency in areas where pellagra is endemic. Lesions characteristic of the disease are seldom seen in infancy but frequently appear in childhood. In the absence of typical lesions the use of nicotinic acid or other active compounds offers a means of confirming a diagnosis of latent pellagra. Frontali (1938) has also shown that when children fed pellagra-producing diets are given ascorbic acid, carotene, riboflavin, aneurin, and nicotinic acid, only those getting the nicotinic acid showed immediate improvement. The cutaneous and mucous lesions healed rapidly and the nervous manifestations vanished in three weeks. Cleckley, Sydenstricker and Geeslin (1939) have reported the beneficial effect of nicotinic acid in the treatment of atypical psychotic states. Katzenellenbogen (1939) working in Palestine brought about considerable improvement in 21 out of 24 cases of stomatoglossitis characterized by soreness of the tongue and angles of the mouth and sore throat. Landor (1939), however, could not cure stomatitis with nicotinic acid but did find yeast to be active. It is quite possible that these conditions may be related to the cheilosis which Sebrell and Butler (1938a) have shown to be due to a riboflavin deficiency. Selfridge (1939) has found nicotinic acid effective in restoring degenerative processes involving the auditory nerves.

The amount of nicotinic acid needed for the treatment of pellagra varies considerably. As little as 50 mgm. may be effective while in other cases 500 to 1000 mgm. per day may be required. Part of this variation may be related to the degree of assimilation, but it is more likely related to multiple deficiencies which will be discussed later.

Since nicotinic acid has been used at rather high levels, studies on its toxicity become important. The work of both Chen, Rose and Robbins (1938) and Unna (1939) indicates the very low toxicity of nicotinic acid and its derivatives. Sodium nicotinate showed a toxicity in mice and rats only when fed at levels ranging from 4 to 7 grams per kilogram body weight. The amide was found to be somewhat more toxic than the sodium salt. Unna found that prolonged oral administration of 2 grams per kilogram daily of sodium nicotinate to rats, chickens, and dogs over periods up to 2 months failed to produce toxic symptoms. The work of Chen, as well as that reported by Elvehjem, Madden, Strong, and Woolley (1938) showed that dogs receiving 2 grams of nicotinic acid per day for several days exhibited some toxicity. However, Unna suggests that the toxicity may have been due to the acidity of the nicotinic acid since he observed no ill effects with the neutralized compound in even larger doses. In any case there seems to be the same wide range between the therapeutic dose and the toxic dose for nicotinic acid as for the other vitamins.

In addition to the above results practically all investigators have found that the administration of large amounts of nicotinic acid to human beings is generally followed by sensations of heat and tingling of the skin. This feeling is accompanied by flushing and rise in skin temperature. At the peak of the flushing Spies, Cooper, and Blankenhorn (1938) found no effect on blood pressure, temperature, or respiration. Sebrell and Butler (1938) studied the quantity of nicotinic acid necessary to produce the unpleasant reactions by dividing a group of 18 normal women into 3 groups and feeding six, 50 mgm., six, 30 mgm., and six, 10 mgm. of nicotinic acid daily in aqueous solution added to tomato juice. On the twelfth day of administration one of the subjects receiving 50 mgm. daily showed an intense flushing of the face, chest and back, which appeared in from ten to fifteen minutes after administration and disappeared after about an hour. Each daily dose produced a similar reaction for eleven successive days until the dose was given in divided portions. Four of the six women on the 50 mgm. level, only two of those on the 30 mgm. level, and none of those on the 10 mgm. level showed these symptoms. The variation among individuals is probably related to rate of adsorption since intravenous injection of 10 mgm. produces a reaction within one minute. Sebrell and Butler conclude that although the reactions are disagreeable, they persist for only a short time and cause no noticeable harm, and therefore their occurrence should not be allowed to interfere with the therapeutic use of large doses of nicotinic acid.

The activity of a number of related pyridine derivatives in black tongue was determined by Woolley, Strong, Madden, and Elvehjem (1938). The active and inactive compounds are listed in table 1. It was immediately apparent that a rather specific structure is required for anti-black tongue potency. The alpha and gamma isomers of nicotinic acid (picolinic acid and isonicotinic acid) are completely inactive. All the compounds listed in which one of the ring hydrogens had been substituted by a methyl or a carboxyl group or in which a methyl group had been added to the ring nitrogen were inactive. The replacement of the carboxyl group of nicotinic acid by a sulfonic acid group or by a cyano group or the removal of the carboxyl entirely (i.e., pyridine) led in each case to inactive compounds.

TABLE 1

Anti-black tongue activity of various pyridine derivatives

ACTIVE	INACTIVE
Nicotinic acid	Pyridine
Nicotinic acid amide	Picolinic acid
Ethyl nicotinate	Isonicotinic acid
Nicotinic acid N methyl amide	Nipecotic acid
Nicotinic acid N diethyl amide	6-Methyl nicotinic acid
β-Picoline	Trigonelline
Nicotinuric acid	1-Methyl nicotinic acid amide chloride
	Quinolinic acid
	β-Aminopyridine

It appears that in addition to the acid and its amide only those compounds possess anti-black tongue potency which are capable of oxidative or hydrolytic conversion to these substances in the body. β-picoline, which might be expected to be oxidized to nicotinic acid, showed a fair degree of activity. Nicotinuric acid was also active, which indicates that the body can hydrolyze this dipeptide. Subbarow, Dann, and Meilman (1938) reported in a preliminary note that β-amino pyridine was highly active in the treatment of black tongue. However, Strong, Madden and Elvehjem (1938) were unable to demonstrate any activity and in a later note Subbarow and Dann (1938) also found β-amino pyridine to be inactive.

It is interesting that there is a close correlation between the results obtained with dogs and those reported by Dorfman, Koser and Saunders (1938) with the dysentery bacillus. Since they found β-picoline to

be completely devoid of growth-promoting activity, the organism evidently cannot oxidize the methyl group as readily as the animal body can.

Many of the compounds found active in the dog have been used in the human. Spies, Bean and Stone (1938) found nicotinic acid, nicotinic acid amide and sodium nicotinate active, diethyl amide of nicotinic acid (coramine) somewhat active, and trigonelline inactive. Sydenstricker et al. (1938) also found the activity of coramine somewhat variable. Ammonium nicotinate and ethyl nicotinate have also been found useful. Recently Vilter and Spies (1939) reported the activity of quinolinic acid in seven patients when fed at 1,000 mgm. levels. Quinolinic acid was found to be completely inactive in the dog when given orally, and since the Vilter and Spies report, McKibbin and Elvehjem (unpublished data) have tried large doses by injection with no improvement in dogs suffering from black tongue. Perhaps the human has a greater ability to decarboxylate quinolinic acid to nicotinic acid than the dog has.

Bills, McDonald and Spies (1939) have also reported the anti-pellagric activity of pyrazine 2,3 dicarboxylic acid and pyrazine monocarboxylic acid. The activity of samples of these compounds supplied by Doctor Spies has been determined with dogs in the reviewer's laboratory. In both cases some response was obtained, but the activity was much less than with nicotinic acid. It was also interesting to observe that the best response was obtained when the dogs did not show the severe symptoms and when the dog was given the compound for the first time. Dogs that had been treated with the pyrazine compound twice in succession showed no response upon the third treatment. These results suggest that the pyrazine compounds cannot take the place of nicotinic acid in all functions but may liberate nicotinic acid from certain non-essential reactions and make it available for the more important functions.

Schmelkes (1939) has prepared thiazole-5-carboxylic acid which is isoteric with nicotinic acid, and found it to stimulate the growth of dysentery bacilli. Its order of activity was one-thousandth of that of nicotinic acid. A sample of this material submitted by Doctor Schmelkes was tested in our laboratory and found to have very slight activity in the dog.

At this point we might ask, is nicotinic acid the true antipellagra factor? The answer to this question depends upon our definition of pellagra. If pellagra is defined as the disease resulting from an insufficient intake of nicotinic acid or related compounds, then nicotinic

acid is the true antipellagra factor. Spies, Grant, Stone and McLester (1938) conclude that nicotinic acid is a vitamin and that pellagra results, at least in part, from a dietary deficiency of nicotinic acid or some closely related substance. Sebrell and Butler (1939) also suggest that in order to avoid further confusion the diagnosis of pellagra should be confined to that syndrome which responds to nicotinic acid. If pellagra includes all the symptoms usually associated with a pellagrin, then nicotinic acid is only one of a group of factors essential for the cure of pellagra. In reality this differentiation is only of academic interest and a question which arises in connection with all our synthetic vitamins. The condition generally found in the field is a multiple deficiency and it is the duty of the medical worker to clear up all the deficiencies. However, in most cases nicotinic acid is the critical deficiency and it must be provided before any significant response can be obtained. Foods carrying only fair amounts of nicotinic acid may be useless in treating severe pellagra regardless of the other factors which they may carry. The difficulties encountered in treating pellagra before nicotinic acid was available substantiate this conclusion. Similar results have been obtained in the case of dogs (Elvehjem, 1939). When a single dose of wheat germ, powdered milk, or dried grass is given to a dog suffering from black tongue, no improvement is obtained. The animal is unable to digest the food sufficiently to liberate the nicotinic acid present. Foods that are richer and can be fed in smaller quantities, such as liver, kidney, lean meat and yeast, work much better.

It would, of course, be much more logical to prevent the development of pellagra than to treat it after it is apparent, but as long as severe pellagra is encountered, the use of nicotinic acid or a related compound will probably be continued. When such a treatment is used, we must remember that after the nicotinic acid deficiency is compensated for, other deficiencies may still occur—especially those due to a lack of the other members of the vitamin B complex. Many of the workers that have used nicotinic acid have observed that the peripheral nerve involvement of endemic pellagrins is relieved only by vitamin B_1. Spies and Aring (1938) have discussed this problem in detail. Both Sebrell and Butler (1938a) and Vilter, Vilter and Spies (1939) have observed riboflavin deficiency in persons ingesting over a period of time a grossly inadequate diet similar to those consumed by pellagrins. It is also now common practice to supplement the Goldberger ration for production of black tongue in dogs with thiamin and riboflavin.

The importance of vitamin B_6 as a supplement to nicotinic acid in

the treatment of pellagrins is not so clear. For a time vitamin B_6 was confused with the antipellagra factor, but Birch, György and Harris (1935) in a comprehensive paper demonstrated that vitamin B_6 was distinct from the anti-black tongue factor (nicotinic acid) and chick antidermatitis factor (pantothenic acid). Dann (1936) showed that vitamin B_6 deficiency in a rat was etiologically different from pellagra. Then there appeared a series of papers on the isolation of crystalline vitamin B_6 (Lepkovsky, 1938; Keresztesy and Stevens, 1938; György, 1938; and Kuhn and Wendt, 1938) and with the availability of the pure vitamin it was easy to show that it had no action in uncomplicated nicotinic acid deficiency. The opinion has been rather prevalent that vitamin B_6 would not play an important rôle in conjunction with nicotinic acid because the pellagrin's diet is high in corn and corn has been considered to be high in vitamin B_6. However, recent work by Black, Frost and Elvehjem (1939) indicates that corn may be very low in vitamin B_6. Spies, Bean and Ashe (1939a) found some beneficial effect of vitamin B_6 in humans. Some of the anemia observed in dogs kept on the Goldberger diet for long periods of time (Miller and Rhoads, 1933; and Spies and Dowling, 1935) may have been related to a vitamin B_6 deficiency since Fouts, Helmer, Lepkovsky and Jukes (1937) and McKibbin, Madden, Black and Elvehjem (1939) have shown that dogs develop a microcytic anemia on diets low in this factor.

The close relationship between nicotinic acid and pantothenic acid both in chemical properties and distribution has already been referred to. To date pantothenic acid has not been available for trial in human cases. However, there are certain animal experiments which suggest the importance of pantothenic acid as well as other members of the B complex.

Dogs grow very well on a modified Goldberger diet when supplemented with thiamin, riboflavin, and nicotinic acid, but if the corn in such a diet is replaced by sucrose the dogs will grow for only a short time and then begin to lose weight. The addition to the ration of 2 per cent of liver extract renders the diet complete. McKibbin, Madden, Black and Elvehjem (1939) found that a concentrate made from liver extract containing mainly factor W and pantothenic acid would supply the necessary factors when synthetic vitamin B_6 was also added. Similar results have been obtained with rats. Helmer and Fouts (1938) found that when rats on black tongue-producing diets were given nicotinic acid they grew less than when they were given the unsupple-

mented diets. Thus nicotinic acid may be the main deficiency in pellagra, but, depending upon the amount of corn used in the diet, any one of the following vitamins may be limiting: vitamin B_1, riboflavin, vitamin B_6, pantothenic acid and factor W.

Mickelsen, Waisman and Elvehjem (1938) placed chicks on a modified Goldberger diet. They grew very poorly but none of them showed any symptoms analogous to pellagra. When this ration was supplemented with nicotinic acid at a level to supply about 1 mgm. of nicotinic acid per chick per day, slightly better growth was obtained. Further work (Waisman and Elvehjem, unpublished data) has shown that a more definite response is obtained with nicotinic acid when thiamin, riboflavin, and vitamin B_6 are supplied in the basal. The addition of a factor U concentrate gives a further growth response above that obtained with nicotinic acid.

Sydenstricker, Schmidt, Geeslin and Weaver (1939) state that from their experience no substance so far tried is as rapidly curative for all manifestations of pellagra as the Cohn fraction of liver extract for intravenous use. This would give further evidence for the value of the other factors associated with nicotinic acid in a natural substance such as liver extract. However, these workers state that it was not possible to demonstrate the presence of free nicotinic acid though nicotinic acid amide might be present in small amounts. Until more accurate chemical methods are available for the estimation of nicotinic acid it is necessary to question the absence of nicotinic acid in liver extract. The preparations tested in our laboratory on dogs have all shown considerable amounts. Evidence which will be presented later suggests that most of the biological activity of liver can be ascribed to free nicotinic acid or its derivatives.

Figures for the distribution of nicotinic acid in natural foods are still rather limited. The values published by Sebrell (1934) gave the anti-pellagra activity of a limited number of foods. After the recognition of nicotinic acid many attempts have been made to obtain more quantitative figures for the distribution of this vitamin. Possible methods include chemical procedures, bacterial growth methods and animal assays.

The early results of Karrer and Keller (1938) based on the color produced with 2.4 dinitrochlorobenzene were definitely too low and in a later paper they (Karrer and Keller, 1939) give higher values and point out that the previous results were too low due to incomplete extraction. Vilter, Spies and Mathews (1938) have used a method

based on the same principle for the estimation of nicotinic acid in urine. The method which appears to be most satisfactory depends upon the breakdown of the pyridine nucleus with cyanogen bromide and aniline to give a yellow colored compound which can be measured colorimetrically. Swaminathan (1938) used this method on foods; Shaw and McDonald (1938) on liver extracts and Pearson (1939) on blood. Von Euler et al. (1938) used β-naphthylamine hydrochloride in place of aniline, but according to Pearson (1939) the method is four times as sensitive when aniline is used. Bandier and Hald (1939) have used p-methyl aminophenol in place of aniline and Bandier (1939) has applied this method to biological materials with satisfactory results. Askelöf and Holmberg (1939), Ritsert (1939) and Kringstad and Naess (1939) have studied further improvements. This method has been studied rather extensively in our laboratory but completely satisfactory results have not been obtained.

The bacterial growth methods have been used for the quantitative estimation of nicotinic acid in body fluids but no extensive studies have been made on foods. As far as animal assays are concerned, both the rat and the chick assays are eliminated at least for the time being. Elvehjem, Waisman and Axelrod (1939) have described the determination of nicotinic acid dependent upon the growth response obtained with standard amounts of nicotinic acid in black tongue dogs. In table 2 are summarized some of the results which have been obtained by the different methods.

As soon as the nutritional significance of nicotinic acid was recognized, it was generally assumed that its function in the animal body must be related to coenzymes I and II. However, it was not easy to obtain direct evidence for this relationship. Both coenzymes are very important in carbohydrate metabolism and they are supposed to differ in structure only by one molecule of phosphoric acid, yet they possess remarkable specificity in relation to the dehydrogenase with which they will react. In most cases a substrate together with its dehydrogenase will react with one of the coenzymes but not with the other. The quantitative estimation of the amount of coenzyme present in tissues is based upon this specificity.

The most obvious approach to any study on the function of nicotinic acid was therefore the estimation of the coenzyme content of the tissues during nicotinic acid deficiency. Von Euler and co-workers (1938) used this approach on rats but unfortunately a specific nicotinic acid deficiency was not produced in the rats. Recently von Euler and co-

TABLE 2

Nicotinic acid content of various tissues

	Mgm. per 100 grams					
Liver, fresh:						
Pig	11.8	(2)	26.5	(10)		
Beef	17.8	(3)	9.3	(5)	25.0	(10)
Sheep	12.5	(4)	47	(10)		
Dog	7.8	(9)				
Kidney, fresh:						
Pig	6.8	(2)	15.6	(10)		
Beef	19.4	(5)	16.9	(10)		
Dog	3.8	(9)				
Muscle, fresh:						
Pig heart	5.3	(2)	8.0	(10)		
Pig	4.7	(2)	3.3	(3)	10.0	(10)
Beef	4.9	(3)	3.8	(5)	10.0	(10)
Beef heart	5.9	(2)	5.1	(10)		
Salmon (canned)	6.0	(3)				
Blood:						
Pig	0.47	(6)				
Sheep	0.83	(6)				
Liver extracts:						
Powder	9–49	(7)	270	(8)		
Liquid	6–122	(2)				
Yeasts:						
Baker's dry	25.7	(1)	12.0	(5)	50.0	(8)
Baker's moist	11.0	(3)				
Brewer's dry	44.7	(3)	59.8	(4)	30–92	(8)
Brewer's moist	10.2	(3)				
Bottom dry	35.6	(1)				
Wheat:						
Whole	5.3	(4)				
Bran	5.0	(3)				
Germ	4.2	(3)	4.0	(8)		
Corn:						
Whole	1.3	(3)				
Peanut meal:						
Raw	13.0	(8)				
Extracted	16.6	(4)				
Soy bean:						
Meal	4.8	(4)				
Skim milk:						
Powder	10.5	(4)	4.3–6.2	(8)		
Eggs:						
Yolk, dry	4	(8)				
White, dry	2.5	(8)				

(1) Bandier and Hald (1939)
(2) Bandier (1939)
(3) Kringstad and Naess (1939)
(4) Swaminathan (1938)
(5) Karrer and Keller (1939)
(6) Pearson (1939)
(7) Shaw and MacDonald (1938)
(8) McKibbin and Elvehjem (Unpublished data)
(9) Ritsert (1939)
(10) Waisman, Mickelsen and Elvehjem (Unpublished data)

workers (1939) found that the tissues from rats on diets low in the B complex tend to be lower in nicotinic acid amide and cozymase than those from normal rats. However, it remains difficult to demonstrate an uncomplicated deficiency of the pyridine nucleotides or any of their precursors in the rat.

Axelrod and Elvehjem (1939) have studied the cozymase changes in dogs and pigs on deficient diets. No changes could be detected in the blood but significant decreases were observed in the liver and muscle. The brain tissue and kidney cortex maintained a normal content under the conditions of the experiment. Kohn, Klein and Dann (1939) found similar results. In dogs with acute black tongue the concentration of coenzyme-like substances (V-factor) was 70 per cent lower in the liver and 35 per cent lower in striated muscle, compared with dogs receiving liberal amounts of nicotinic acid. The decreased coenzyme content of the liver in black tongue was accompanied by an increase of 35 per cent in oxygen consumption. The kidney coenzyme content was unchanged but its ability to oxidize lactate was decreased by 50 per cent.

Studies on the blood of normal and pellagrous patients have not given completely comparable results. Using the method of Lwoff, Kohn (1938) found no significant difference between the blood of normals and pellagrins but did find an increase even in normal patients upon administration of nicotinic acid. Vilter, Vilter and Spies (1939) however, found that the blood of normal persons supports growth of B. influenzae to a much greater extent than the blood of pellagrins. After nicotinic acid therapy, the blood of the pellagrins increased to normal growth promoting activity. In a more extensive study Kohn and Bernheim (1939) have determined the V-factor (coenzymes I and II plus possibly unknown related compounds) in the blood of 53 normal individuals and 126 hospital patients. Eighty-one per cent of the normal cases fell between 50 and 80 d.c. units (equivalent to gamma of coenzyme per one ml. of corpuscles). Only 65 per cent of the pathological cases fell in this range. Pellagrins showed values that were in the lower part of the normal range. They concluded that such determinations can be of little value in the diagnosis or prognosis of pellagra. Ballif, Lwoff, Querido and Ornstein (1939) found no reduction in the nicotinic acid amide content of the blood of 10 cases of pellagra. Axelrod, Gordon and Elvehjem (1939) found 20 to 30 micrograms of cozymase per milliliter of blood in normal individuals and the value increased to 50–60 micrograms upon the ingestion of 100 mgm. nicotinic acid per

day. When the nicotinic acid was no longer supplied the cozymase in the blood gradually returned to normal value during a period of about two weeks. Kohn and Klein (1939) have been able to establish that the human erythrocyte can synthesize V-factor from nicotinic acid in vitro as well as in vivo. Since this synthesis is accompanied by an increased ability to oxidize lactic acid, they conclude that cozymase has been synthesized.

Whether the changes observed in the liver and muscle of nicotinic acid deficient animals also occur in humans and if this decrease is sufficient to account for the gross symptoms observed cannot be answered without further information. However, it is quite possible that at least part of the rapid improvement noted both in humans and animals when nicotinic acid is administered is due to the rapid formation of cozymase when the nicotinic acid part of the molecule is made available.

Elvehjem, Waisman and Axelrod (1939) have found that if the cozymase content of fresh animal tissues is converted to the equivalent amount of nicotinic acid, it makes up an appreciable amount of the total nicotinic acid determined by animal assay. Part of the nicotinic acid must be present as coenzyme II and there is undoubtedly some free nicotinic acid and amide. More definite comparison can be made when the chemical methods are accurately standardized.

A certain amount of information has been gained from studies on the urine during nicotinic acid deficiency. Fraser, Topping and Sebrell (1938) used a method for measuring nicotinic acid or related compounds in the urine based on the growth promoting properties with Shigella paradysenteriae (Sonne). No attempt was made to estimate the nicotinic acid quantitatively. They did observe a marked increase in the growth promoting power of the urine following the administration of nicotinic acid as well as a marked decrease in the case of urine from dogs with black tongue. There was a close correlation between the biological assay of the urine and the clinical condition of the animal.

Spies, Bean and Stone (1938) report a tremendous variation in the nicotinic acid content of the urine from the same individual from time to time and from person to person. The amount excreted, however, was dependent upon the diet and the amount of nicotinic acid given and the mode of administration. A pellagrin in relapse retains more of the administered acid than does a normal person of the same size. Spies, Bean and Ashe (1939) also found an increase in the concentration of coenzyme in the urine within 24 to 48 hours after giving nicotinic acid.

Another interesting study has been the relation of nicotinic acid to pigment excretion. Beckh, Ellinger and Spies (1937) reported an increase in the amount of coproporphyrin I or III in the urine of pellagrins and a decrease following the treatment of the pellagra with yeast or liver extract. Later Spies, Gross, and Sasaki (1938) found that nicotinic acid also produces a decrease of porphyrinuria in pellagrins. They also found that the porphyrinuria associated with other diseases promptly decreased following nicotinic acid therapy. Watson (1938) found that the urinary copoporphyrin in alcoholic pellagrins was not correlated with the Beckh-Ellinger-Spies test. In a later paper Watson (1939) concludes that the color reaction observed in the urine during pellagra is due to urorosein. It occurs as a chromogen which changes to a pink or red pigment upon addition of hydrochloric acid to the urine. Another pigment soluble in chloroform may also be present which appears to be indirubin. Both of these pigments may be noted in the urine of patients not having clinical pellagra and therefore further work is necessary in order to relate the appearance of these pigments directly to nicotinic acid deficiency. Meiklejohn and Kark (1939) have also found substances in the urine of pellagrins capable of giving the urorosein reaction. They were unable to find any unusual amount of coproporphyrin in four samples of urine supplied by Doctor Spies and suggest that it would be more proper to refer to the Beckh-Ellinger-Spies test as indicating the presence of pigments capable of producing the urorosein reaction.

In summary we may emphasize two important facts which apply not only to nicotinic acid but to the vitamins in general. One is the importance of the experimental animal in extending our knowledge of important nutritional factors as well as fundamental metabolism. In the case of nicotinic acid the species of animal used has been an important factor. This does not mean that the fundamental metabolism in different species varies greatly, but rather that specific deficiency is produced more easily in one species than in another. Thus the rat was used successfully in separating the antipellagra factor from thiamin, the chick for separating it from riboflavin and the dog for establishing its relation to nicotinic acid and not to the chick antidermatitis factor. When the specific information is obtained with the animal it is readily transferred to the human.

The second is the importance of recognizing the specificity of each of the individual vitamins. Each vitamin has a very limited effect on the animal body and can be used with success only when it is lacking

in the diet. As more of the water soluble vitamins are obtained in crystalline form, it is more evident that additional factors must be recognized. The full value of one factor can only be realized when it is used in conjunction with all the essential factors. Thus nicotinic acid will cure or prevent all conditions produced by a true nicotinic acid deficiency but can be of no value in the treatment of conditions produced by a deficiency of other factors and its value in the treatment of pellagrins is much more effective when all other factors are supplied in optimum amounts.

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THE PHYSIOLOGY OF ARTICULAR STRUCTURES[1, 2]

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In contrast to numerous detailed descriptions of the anatomical features of joints, few comprehensive studies of their physiological properties are available. The statement made by Bichat (33) in 1799, that "no part of the physiology of the skeleton abounds more in hypothesis and less in discoveries than the synovial system," still holds a considerable element of truth. Yet today, articular disorders rank foremost among the crippling diseases. This challenge cannot be met without gaining a better insight into the physiological principles upon which the function of joints is based. Although many questions concerning the growth and activity of articular structures remain as yet unanswered, it is hoped that this presentation will facilitate a review of the available experimental evidence and stimulate further investigation. A summary of the development and anatomy of articulations will assist in correlating form and function. The physiological significance of the articular elements will be considered separately and their close interaction discussed.

ARTICULAR STRUCTURES. Articulations develop according to an inherited pattern, although their anatomy may be altered by ontogenetic influences (31, 41, 169, 214). In human embryos, the anlage of most joints is complete at the beginning of the third fetal month (157). It consists, typically, of two chondrogenous zones with an interposed remnant of mesenchyme. In the latter, liquid hyaloplasm appears (169, 213, 231, 239), probably elaborated by the mesenchymal cells (171, 269). These retract peripherally to enclose with their densely packed fibrils the future joint cavity, thus forming the embryological equivalent of the synovial membrane.

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The nature of the tissue lining joint spaces has been widely debated, but much of this discussion is of historical interest only. Suffice it to say that it has been claimed to be simple (123), stratified (236), glandular (121, 280) epithelium, single (126) or multilayered (151, 258) endothelium, fixed connective tissue (5, 33, 37, 40, 78, 110, 111, 175, 267), metaplastic cartilage (169), a reticulo-endothelial organ (88) and specialized connective tissue (176, 269). To Hueter (132) we are indebted for a fundamental description of the synovialis as a connective tissue structure, which, in essence, still holds. Microscopically, it appears as a substantial membrane of variable thickness, whose inner aspect shows a smooth, glistening surface, particularly where it overlies ligaments, tendons and menisci, while in other areas it has a dull lustre. Plicae or folds are numerous and occasional trabeculations occur, forming partially closed off compartments. An inner layer, the intima, can be lifted off easily in some parts, but not in the peripheral zone and over the tendons and menisci, where it adheres firmly. The intima may extend for a short distance onto the marginal cartilage as it does regularly in human embryos, while the outer fibrous layer forms a less complete covering, blending with the perichondrium and periosteum. Microscopically, two layers are again distinguishable, but it is readily apparent that there is no definite line of demarcation, and that the degree of differentiation is more marked in certain areas. The intimal cells near the articular surface are large, round or polygonal, with coarsely granular cytoplasm. Their nuclei are oval and their nucleoplasm shows a chromatin network. The cellular arrangement is single or multilayered, simulating endothelium or cuboidal epithelium, but there is no regularity of such patterns and a basement membrane has never been demonstrated. The synovial lining cells have protoplasmic processes which extend both parallel to the surface, where they interlace and anastomose with those of other cells, and into the deeper fibrous layer. The structure of the intima varies considerably in different regions of the membrane. Hammar (111) distinguished a cellular and a cell-poor type while Franceschini (88) contrasted a simple, acellular, poorly vascularized form closely related to the underlying fibrous stratum, with one in which he found histological characteristics of the reticulo-endothelial system. Occasionally, cells or cell groups are seen lying on the surface. More commonly, the cellular elements are separated from the cavity by collagenous material of varying thickness. Elastic fibres are scarce in the intimal layer. In the deeper fibrous stratum, the cells are smaller and have fewer, blunter and shorter

processes. The principal difference lies in the predominance of the intercellular substance. Broad bands of collagen and a slightly greater number of elastic fibres are present. Adipose tissue, of which there is a generous amount in the subintimal layer, particularly in early life (89), diminishes in the outer part of the capsule. From both the cellular and the cell-poor areas of the synovial surface, but more frequently from the former, villi of various sizes project into the joint cavity. The presence of blood vessels in the articular membrane was first demonstrated by Hunter (134) who described the "circulus articuli vasculosus". Subsequently, the vascular supply of the synovial membrane was studied histologically (37, 40, 110, 111, 176) and by arterial injection (172, 220, 259). A well-marked vascularity of the synovialis was ascertained and the capillaries were found to extend through the fibrous layer close to the synovial surface. Böhm (37) and others (164, 170, 260) failed to demonstrate lymphatic channels by intra-articular injection and disputed von Mosengeil's contention (183) that well-defined stomata connected the articular cavity with the lymphatic system. While Hueter (132) made the first definite histological observation of lymphatics in synovial tissue, Tillmanns (260) could, by direct injection of silver nitrate into the membrane and a counter-injection of the vascular tree, demonstrate a subintimal and a deeper plexus of lymphatics. Much confirmatory evidence has since been presented (21, 57, 83, 158, 172, 185, 186, 284). The finding of nervous tissue elements in the synovialis has been reported with great unanimity (65, 111, 151, 155, 192, 196, 209, 227, 247, 256). In a comprehensive study, Gerneck (95) reported the existence of a myelinated ground plexus and a superficial plexus of non-myelinated fibres. The type of endorgans indicated that both vasomotor and sensory functions are served.

Recently some workers (88, 159, 176, 228, 270) have suggested that the synovial intima represents connective tissue adapted to specific functions such as elaboration of synovial fluid constituents and reticulo-endothelial activity. King (147) claimed to have demonstrated Golgi material in the cytoplasm of the synovial cells. While interesting perspectives have been disclosed in these studies, they rely largely on disputed staining methods. Other evidence is required to lift them out of the realm of speculation.

With few exceptions, the articular surfaces of bone are covered by hyaline cartilage. Grossly, this is of bluish white, ground-glass appearance. Its structural components are the typical cartilage cells housed in lacunae, an intercellular system of fibrils and the undiffer-

entiated hyaline matrix. The preponderance of matrix over cells is striking. Superficially, the cells are flat and lie parallel to the joint surface. In the subjacent, transitional layer, the cells are irregular, the lacunae larger and grouped together. The cell processes exhibit branchings and form plexuses. In a third zone, the cells are round and large, with wide, blunt processes, and arranged in rows perpendicular to the articular surface. The lowermost layer extends to the line of demarcation beyond which the matrix is calcified. Adult human cartilage is avascular (134) and has not been shown to contain lymphatics or nervous tissue (77, 111, 125, 260).

Some joints are supplied with menisci and labra. The structure of the latter resembles that of the synovial membrane, and metaplasia to fibrocartilage is common. Tendons may be intimately associated with the joint capsule, so that, at times, they take the place of the fibrous stratum. Bursae are frequently connected with the joint cavity proper, or located as separate structures between tendons and bony prominences. Their embryological derivation from a mesenchymal cleft and the histological character of the bursal wall suggest a close relationship with synovial tissue (140).

In summary, then, it may be stated that a typical diarthrosis consists of the opposing articular cartilages, the capsule connecting them and a variable amount of synovial fluid. Embryologically and histologically, the articular components are of mesenchymal character. The membrane conforms in structure to connective tissue. It possesses a collagenous matrix, a rich supply of blood vessels, lymphatics and nerves, few elastic fibres and characteristic connective tissue cells. The cellular elements are sparse in the external layer, but near the lumen cell aggregates occur, representing a modification of fixed connective tissue cells. Direct communications between the lymphatics of the membrane and the articular cavity have not been demonstrated. On the other hand, no apparent barrier exists separating the articular cavity from the intercellular spaces of the synovialis. The evidence presented indicates that the joint is a tissue space rather than a body cavity. The designation of synovial tissue as a membrane, although in common use, is anatomically incorrect and should be employed with this understanding.

ARTICULAR FUNCTION. Our knowledge of the structure of joints, although far from complete, exceeds considerably that of joint function because the interest has only in recent years shifted from morphological to physiological research.

Earlier workers were concerned with the forces which hold joints together. The Webers (276) claimed to have proven that it is the atmospheric pressure, rather than the action of muscles, tendons and ligaments, which saves man from dislocating his femur in the act of walking. Later investigators (94, 234), however, showed that barometric pressure could support merely a fragment of femoral head (35 grams) in the acetabulum and argued correctly that a partial vacuum in a joint cavity, sufficient to carry the weight of an extremity (10–13 kgm.), would be a physiological curio. Allen (7) emphasized the importance of the capsular ligaments, while Fick (78) again stressed the effectiveness of atmospheric pressure. Bordier (39) suggested that the molecular cohesive force of a synovial fluid film aids in maintaining apposition of joint surfaces. This factor, of course, cannot be fully active in extremes of motion, and therefore it is evident that the supportive structures, mainly the ligaments, are in a large measure responsible for the stability of joints.

Cartilage. Articular cartilage is the final recipient of all jolts and blows exerted upon the skeleton. The physical properties by which this tissue attains its efficient buffer action have been investigated in vitro (17, 28, 99, 210) and resilience has been found to be a pre-eminent characteristic. Cartilage is almost completely elastic to frequent intermittent pressures, while continuous compression of the same total strength decreases its expansile power and lengthens the period of recovery. Much of the elastic latitude is lost by drying but returns if the desiccated material is suspended in physiological saline, even after an interval of several months. Benninghof (28) therefore considered that the mechanism of elastic action consists of exudation and reabsorption of fluid. These observations gain further significance when we consider the nutrition of this avascular tissue. In accord with Toynbee (264) and Hunter (134), some workers (45, 283) still attach primary importance to subchondral, capsular or perichondrial blood sources. Others (16, 82, 137, 194) hold that such blood supply serves as a source of nutrition only for the immediately adjacent cartilaginous parts. The prevailing opinion (9, 36, 138, 154, 169, 254) is that the bulk of articular cartilage derives its nourishment from the synovial fluid. Evidence for this has been gained from the study of spontaneous and experimentally created loose bodies in joints. While those consisting of bone undergo necrosis, cartilaginous fragments remain viable for many weeks (26, 114). No conclusive data have been presented elucidating the mechanism by which nutritive material enters and

waste leaves the cartilaginous substance, but it is assumed that metabolites pass along the intercellular system of fibrils (169). Müller (187) conceived of the exchange as a physical process, induced by alternating pressure and suction. Other workers (116, 182) searched for a clue by studying, in vitro, the permeability of cartilage to chemical radicals. Electrolytes supposedly penetrated poorly and without selectivity (116). Hydrogen and hydroxyl ions exhibited even less tendency to diffuse. Nonelectrolytes entered in indirect relation to their molecular size and in direct variance with lipoid solubility. In these experiments the presence of the diffusing substances in cartilage itself was not considered, a factor which would influence the establishment of equilibria.

More definite data have been obtained from studies pertaining to metabolism of cartilage. Its respiration is nearly nil (47, 49, 160) and, like muscle, it possesses the faculty of anaerobic oxidation. The metabolic rate is one-tenth that of connective tissue but, per cell, is of the same order as that of other tissues. Kuwabara (160), confirmed by Bywaters (49), demonstrated the presence of a dehydrogenase (hexosephosphate). Interesting relationships have been revealed between glycogen content, glycolysis and growth of cartilage. Hoffman et al. (127) demonstrated that epiphyseal cartilage of cattle embryos contains nearly 3.5 times as much glycogen as adult tissue or, as expressed by Gendre (93), the glycogen content varies with the state of hypertrophy. Bywaters found the metabolism of fetal epiphyseal rabbit cartilage ten times that of adult cartilage, or double the amount per cell. The higher glycogen content of fetal epiphyseal cartilage could also be demonstrated histologically in specimens stained with Best's carmine (117, 136). In older animals, glycogen was occasionally absent in epiphyseal cartilage. The glycogen content of the epiphyses is smaller and more labile than that of rib cartilage, a finding which would agree with Dickens' (70) report of higher metabolic rates in the latter. In addition to glycogen, articular cartilage is known to contain lactic acid, collagen, chondroitin sulfuric acid and calcium salts. Its exact chemical composition has not been determined with any degree of completeness.

Growth and repair of cartilage are more fully understood. In a carefully controlled histological study on epiphyseal cartilage of mice and rats, Elliott (74) was able to show that mitosis is the normal mechanism of cell division in immature cartilage. As maturity progresses, amitosis prevails and in adult tissue it is the sole form of growth. The area of maximal activity appears to be located a short distance beneath the articular surface. Bennett and Bauer (23) likewise concluded that

following experimental injury, proliferative activity is greater in the deeper zones. Recent experimental work (248, 249, 250) if confirmed will aid in evaluating the rôle played by endocrine factors in the growth of cartilage. Anterior pituitary extract was found to induce hypertrophy and hyperplasia in the transitional zone, followed by liquefaction and ulceration of the articular surfaces, in young guinea pigs, whereas in adult animals the degenerative changes predominated. Similar sequelae were observed after gonadectomy in immature subjects. Oral administration of thyroid extract for from two to thirty days, enhanced growth and differentiation of euhyaline cartilage in normal young guinea pigs. The indispensability for the growth of cartilage of such functional stimuli as pressure and attrition has been repeatedly postulated (29, 40, 77, 187, 225). Bennett, Bauer and Maddock (26) demonstrated that apposition of articular surfaces is required for the integrity of joint cartilage and that proliferative reactions, following experimental trauma, are stronger in weight-bearing than in passive areas. Reyher (214) found that when he immobilized the extremities of animals for sixty-two days, only the non-contiguous joint surfaces showed evidence of degeneration. Proper function, of course, precludes a multitude of pathological states not within the scope of this review. However, the deleterious effect of continuous pressure on joint cartilage as revealed in clinical experiences (58, 153) and experimental studies (157, 224) should be mentioned because it tends to confirm Müller's concept of the nutrition of cartilage. Histological studies have shown that, with advancing age, the superficial zone undergoes fibrillary degeneration, while the vertical stratum moves toward the surface (10, 11, 24, 25). Cataplastic changes were found to begin almost immediately upon cessation of growth.

From the reaction of cartilage to injury, further information concerning its growth has been obtained. Leidy (1849) (167) was the first to observe that experimental cartilaginous fractures did not unite. Redfern (211), two years later, added that repair of cartilaginous wounds may take place by invading fibrous tissue. A host of experimental studies have since confirmed these results, with slight modifications. A few workers found that injuries confined to cartilage showed no evidence whatever of regeneration (96, 107, 108). Somewhat more numerous and, in general, of more recent origin are observations of slight or limited healing in such wounds (15, 56, 202). Häbler (109) reported that superficial cuts remained essentially unchanged for three hundred and four days and that only in one of sixteen specimens was there any microscopic evidence of proliferation. Bennett, Bauer and Maddock

(26) published an experimental study, controlled as to location of injury and age of animals. They observed that experimental lesions in normal young adult dogs showed only slight proliferation after as long as twenty-eight weeks, when the injury did not involve subchondral bone or perichondrium and was located on weight-bearing surfaces. Ultimately, some of these defects were filled in with tissue having an imperfect resemblance to hyaline cartilage. On passive surfaces, no repair at all was observed in two joints after four and twelve weeks respectively. Bennett and Bauer (23) demonstrated that the proliferative ability of joint cartilage in immature animals does not exceed that of grown subjects. Most observers agree that wounds in cartilage extending into the subchondral bone, or located close to the perichondrium or the synovial membrane, fill in rapidly with a fibrous pannus. This, in turn, may undergo metaplasia to fibrocartilage and at times to imperfect hyaline cartilage. Seggel (243), Fasoli (76) and Shands (245) occupy a somewhat different position, emphasizing more strongly the regenerative power of cartilage.

Cartilage thus has been shown to be an elastic, avascular tissue with a low ratio of cells to matrix. Its chief, if not sole, source of nourishment is the synovial fluid. The mechanism by which nutritive material gains entrance has not been established. The low oxygen requirement and limited reparative ability confirm the fact that cartilage is a relatively inactive tissue.

Synovial membrane. The mechanical properties of the articular capsule are determined by its fibrous layer, rather than by the intima. As might be expected from its anatomical structure, the synovial membrane has little elasticity. If fresh capsular tissue is tested in vitro, the elastic resistance is about thirty times that of a sheet of rubber of equal thickness (66). Elastic expansion can therefore account for only a small increase in surface area. Since, in the physiological state, a large portion of the cavity walls is collapsed by the pull of tendons, additional space may be made available through muscular relaxation. Direct determinations of the potential joint volume by instillation of fluids are subject to many errors. The results to date show such wide discrepancies as 60 and 300 cc. for the knee joint of a normal adult male (77, 201). When there are sudden, large accumulations of fluid, actual stretching of the membrane may take place. But like tendinous tissue, it possesses a high resistance to tear (97 kgm. per sq. cm.). The marked pliability, considered by Danckelmann (66) the outstanding physical characteristic, aids in withstanding the severe stresses of joint motion.

The metabolism of the synovial membrane, per cell, was found to be

of the same magnitude as that of other tissues (47). Respiratory quotients varying in the majority between 0.71 and 0.72 suggest predominant oxidation of fat. A positive methylene blue reaction indicated the presence of dehydrogenase.

In contrast to cartilage, the articular membrane has a pronounced and undisputed regenerative ability (142, 242, 282). Key (142), in a comprehensive study, observed a series of hemisynovectomized knee joints of rabbits up to one hundred and four days. After a primary polymorphonuclear reaction in the fibrin clot, clasmatocytes and monocytes appeared and organization by connective tissue was usually complete within six days. A layer of collagen formed on the repaired surface, beneath which fibroblasts were seen in aggregates. On the tenth day, the number of cells in this layer had increased markedly. In the ensuing stage, a diminution of cellular activity paralleled by abundant deposition of collagen was noted. Villi developed and after about sixty days, a nearly normal synovial membrane was present. Restitution had thus taken place from the external or deeper tissues, with little or no growth from the edges. From these and other experimental studies, Key (142, 144) concluded that joint cavities are clefts lined by slightly modified connective tissue cells. Wolcott (282) reported that following typical synovectomy, the excised membrane regenerated completely, both in adult and in young dogs. Thirty to one hundred days postoperatively, the new tissue was nearly indistinguishable from a normal articular capsule in structure, size and contour.

Synovial fluid. Cytology. Normal joints contain varying amounts of synovial fluid. Rarely can one aspirate more than a drop from the knee or shoulder joints of the smaller laboratory animals (rabbit, cat and dog) (216, 275). In young western cattle from 3 to 7 cc. of synovial fluid can be obtained from the carpometacarpal joint, whereas 15 to 40 cc. can be aspirated from the astragalotibial joint (19). The average quantity obtainable from a normal human knee joint is 0.45 cc. with a minimal-maximal variation of 0.13 to 2.0 cc. (60).

The earliest reports (111, 161) indicated that normal synovial fluid is relatively cell-poor but gave little information as to the percentage of each cell type. Subsequent studies (19, 144, 275) agreed as to its cell content but revealed marked variations in the total number of cells and the percentage number of any one type in the various species of animals. Key (144) working with rabbits found that shoulder joint fluid contained 175 to 225 cells per cu.mm. and approximately the same number of erythrocytes. By means of supravital staining, he

established the following differential nucleated cell count: monocytes, 58 per cent; clasmatocytes, 15 per cent; indeterminate phagocytes, 14 per cent; leukocytes, 5 per cent; primitive cells (resembling small lymphocytes), 1 per cent, and synovial cells, 3 per cent. Warren, Bennett and Bauer (275) found somewhat higher total nucleated cell counts in fluid from the knee joints of normal rabbits but the average differential cell count was approximately the same. These same workers (220, 275) reported average nucleated cell counts of 112, 131 and 182 cells per cu.mm. in three large series of astragalotibial joint fluids and total counts of 213 and 222 cells per cu.mm. in two series of carpometacarpal fluids (cattle). The average nucleated cell count for astragalotibial fluid was: monocytes, 36.4 per cent; clasmatocytes, 15 per cent; unclassified phagocytes, 3.9 per cent; lymphocytes, 40.1 per cent; polymorphonuclear leukocytes, 2.2 per cent; synovial cells, 1.2 per cent, and unclassified cells, 1.2 per cent. The differential cell counts on the more cellular carpometacarpal fluid averaged: monocytes, 63 per cent; clasmatocytes, 7.2 per cent; unclassified phagocytes, 3 per cent; lymphocytes, 23 per cent; polymorphonuclear leukocytes, 1.2 per cent; synovial cells, 1.7 per cent, and unclassified cells, 1 per cent. It was thought that cellular debris associated with the regularly occurring cartilage defect in the carpometacarpal joints was sufficient stimulus to cause the increase in the total number of nucleated cells and the higher percentage of mononuclear phagocytes. The highest average nucleated cell count (963 per cu.mm.) was observed in the synovial fluid specimens obtained from dogs (275). The average differential nucleated cell count of the dog fluids was very similar to that of the carpometacarpal fluids of cattle. Erythrocytes were absent in many of the synovial fluid specimens obtained from the various species of animals (19, 275), thus indicating that they are not normal constituents of synovial fluid. When found, they are undoubtedly the result of trauma incident to the aspiration. Neither the total number of nucleated cells nor the individual cell types are influenced by variations of the blood cytology (19). Post mortem migration of cells into the joint may take place but occurs without any appreciable alteration in the percentage of individual cell types (275).

Knowledge of normal human synovial fluid cytology was very meagre until recently because no one of the previous workers (86, 111, 144, 148, 161, 177) had examined more than one or two fluids. A study of twenty-nine fluids obtained postmortem from human knee joints showing no evidence of disease has been made in our laboratory (60). The

total number of nucleated cells varied from 13 to 180 cells per cu. mm., the average being 63 cells per cu.mm. The total cell counts on knee joint fluids from the same individual in most instances were approximately the same. Again it was found that the synovial fluid cytology was never a reflection of the blood cytology. Failure to detect erythrocytes in eight of these fluids serves as further evidence that they are not normally present. The average differential nucleated cell count was: monocytes, 47.9 per cent; clasmatocytes, 10.1 per cent; unclassified phagocytes, 4.9 per cent; lymphocytes, 24.6 per cent; polymorphonuclear leukocytes, 6.5 per cent; synovial cells, 4.3 per cent, and unclassified cells, 2.2 per cent. Eosinophils and basophils were not observed in any one of the twenty-nine synovial fluids examined, indicating that they are rarely present in normal synovial fluid.

The average total cell count of human synovial fluid is lower than that of any animal species thus far studied although marked individual variations were noted. The total cell count in many instances might have been higher if the joints had been subjected to normal use up to the time of death. Again mononuclear phagocytes represented the predominant cell type. From previous studies (19, 275) it would appear that their chief function is the removal of wear and tear products (debris and particulate matter) resulting from minor traumata and insults. That type and degree of trauma influence synovial fluid cytology can readily be demonstrated. Injection of normal saline into a rabbit's knee joint (19) results in an immediate rise in the polymorphonuclear leukocytes which are replaced subsequently by mononuclear phagocytes. Apparently such mild irritation produces sufficient change in the subsynovial capillaries to allow for an immediate intra-articular migration of polymorphonuclear leukocytes, whereas the mononuclear phagocytes arising from the synovial tissue and tissue fluids are much slower to respond (146). Therefore, it seems reasonable to assume that the variations in total number of cells and individual cell types encountered in normal synovial fluid probably represent the intra-articular response to the inconstant trauma incident to daily use. It is of further interest that the highest cell counts are observed in those joints showing the greatest wear and tear changes in consequence of increasing age and long-continued use (24, 25).

Since synovial fluid serves chiefly as a lubricant for the articular surfaces and as a source of nourishment for articular cartilage, it is not surprising that it contains relatively few cells. If the joint cavity is an enlarged tissue space (62, 132, 144, 147), lined by modified connective

tissue, the cytology of synovial fluid may be considered representative of that of tissue fluid.

Physical Characteristics. Complete physical and chemical characterization of normal synovial fluid has been possible in cattle (220), and normal human fluid has been compared in enough cases to indicate that the fluids are essentially alike (222).

Normal synovial fluid is a clear, pale yellow, viscous liquid. Its pressure has not been adequately studied. No satisfactory method which takes into account variations due to motion and muscle tone has as yet been devised. In a moderately well-controlled study, Müller (188) examined the synovial fluid pressure on fresh cadavers, anesthetized humans, anesthetized dogs and on non-anesthetized standing dogs. He concluded that a slightly negative pressure exists in normal joints, ranging from −2 to −12 cm. of water and varying with muscle tone and position.

The average specific gravity of cattle synovia is 1.010 with a maximum of 1.012 and a minimum of 1.009. In human fluids obtained post mortem from joints in which no histological changes were found in the membrane, Horiye (129) found similar figures ranging from 1.008 to 1.015.

The average content of total solids of cattle fluid is 2.08 grams per 100 grams with a maximum of 3.89 and a minimum of 1.67, as compared with an average of 8.73 in the serum. These figures correspond closely with those of Horiye (129) for post mortem human fluids (1.20 to 3.93 per cent), but are slightly lower than the value given by Fisher (83) for normal human fluids obtained post mortem (4.4 per cent).

The average freezing point of cattle fluid is −0.535° with a maximum of −0.556° and a minimum of −0.590°, as compared with an average for the serum of −0.590°.

The relative viscosity of normal synovial fluid varies markedly in different joints. In fluid from the astragalotibial joints of normal cattle, the average is 3.7 at 25°C., with variations from 2.8 to 4.2. Fluid from the carpometacarpal joints, on the other hand, has an average viscosity of 64, with variations from 29 to 129. Normal human fluid has been found to have an even higher viscosity, with an average of 124 and a range from 51 to 209 (222). Schneider (237) reported variations from 3.9 to the unlikely value of 1490 in fluids obtained post mortem from patients without joint disease. Kling (149) found viscosities of 10.7 and 20 in two normal numan fluids. The factor responsible for the high viscosity appears to be mucin. Following precipitation of the

mucin, the viscosity of the fluid approaches that of water. The cause of the marked variations in different joints is not known. They correspond, in general, with the variations in mucin content and, furthermore, seem to be associated with the degree of polymerization of the mucin.

The average osmotic pressure against Ringer-Locke solution is 365 mm. of water for the serum and 150 mm. of water for cattle fluid. By

TABLE 1

Chemical composition of normal cattle synovial fluid and serum

	PROTEIN (NOT INCLUDING MUCIN)		ALBUMIN		A/G RATIO		MUCIN	SUGAR		NON-PROTEIN NITROGEN		UREA		URIC ACID		pH	
	Se	Fl	Se	Fl	Se	Fl		Se	Fl	Se	Fl	Se	Fl	Se	Fl	Se	Fl
	grams	*grams*	*grams*	*grams*			*grams*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*		
Average*	7.87	0.886	3.97	0.712	1.10	3.90	0.138	91	66	24	21	8.5	8.2	1.84	1.55	7.42	7.31
Maximal	8.75	1.410	4.14	0.889	1.27	5.82	0.252	176	93	31	28	12.8	14.1	2.26	2.08	7.55	7.43
Minimal	7.11	0.435	3.64	0.493	0.98	2.48	0.033	58	45	18	15	6.6	6.1	1.54	1.20	7.31	7.17
Number‡	37	36	7	6	7	6	11	12	12	32	30	15	15	7	7	21	23

	CHLORIDE		BICARBONATE		PHOSPHORUS		SULPHATE		LACTIC ACID		TOTAL BASE		SODIUM		POTASSIUM		CALCIUM		MAGNESIUM	
	Se	Fl	Se	Fl	Se	Fl	Se	Fl	Se	Fl	Se	Fl	Se	Fl	Se	Fl	Se	Fl	Se	Fl
	m. eq.	*m. eq.*	*m. eq.*	*m. eq.*	*mM*	*mM*	*m. eq.*	*m. eq.*	*m. eq.*	*m. eq.*	*m. eq.*	*m. eq.*	*m. eq.*	*m. eq.*	*m. eq.*	*m. eq.*	*m. eq.*	*m. eq.*	*m. eq.*	*m. eq.*
Average†	109.8	110.5	26.8	28.5	2.2	2.2	5.56	4.96	5.47	3.21	165.7	163.2	156.1	145.0	5.37	4.04	5.6	3.8	1.75	1.44
Maximal	117.1	116.3	29.5	31.8	3.0	3.0	6.03	5.42	9.71	3.80	180.6	180.8	167.9	147.8	5.87	4.40	6.2	4.3	2.22	1.72
Minimal	103.6	104.9	23.8	25.3	1.4	1.5	5.28	4.53	4.14	2.06	154.2	152.5	148.7	140.1	4.44	3.60	4.6	2.7	1.54	1.33
Number‡	31	31	13	15	15	15	6	6	5	6	18	18	6	6	6	6	15	15	8	8

* All values represent the concentration in 100 grams of water.

† All values represent the concentration in 1000 grams of water.

‡ Represents the number of fluids from which the average figures were obtained.

direct determination the average osmotic pressure difference between the fluid and the serum is 250 mm. of water. It is of interest to compare these values with the colloidal osmotic pressures calculated from the average albumin and globulin figures. Using the factors determined by Govaerts (103) for the pressure exerted per gram by serum albumin (75.4) and serum globulin (19.5), the osmotic pressure of the serum is 384 mm. of water. This agrees fairly well with the observed value of 365 mm. In the case of the fluid, however, the pressure calculated from the albumin and globulin content is only 57 mm. of water, in contrast

to the observed value of 150 mm. Mucin is the only other colloidal substance known to be present in the fluid. The osmotic pressure exerted by mucin has not been determined. If one assumes that the difference between the observed and the calculated osmotic pressures of the fluid is due to mucin, its osmotic pressure effect per gram is nine times as great as that of albumin (675 as compared with 75).

Protein Constituents. The average concentration of protein not including the mucoprotein, is 0.88 gram per 100 cc. in cattle fluid, in contrast to 7.40 grams per 100 cc. in the serum. This figure is in the same range as that found for normal human synovial fluid. In specimens obtained post mortem from patients who had had no joint symptoms and no edema, it was 1.36 grams per 100 cc., with variations from 0.23 to 2.13 grams (222). Fisher (83) reported a protein content of 1.6 per cent in normal human fluid and 0.92 per cent in cattle. Horiye (129) found variations from 0.45 to 3.15 per cent in fluids obtained post mortem from joints in which he found no histological changes in the membrane. Judging from the findings in a large number of normal and pathological fluids, we would conclude that the value of 3.15 per cent represents an abnormal fluid. Cajori and Pemberton (52), in synovial fluid from a patient with generalized edema, found a protein concentration of 1.39 per cent. The protein content reported for other body fluids is somewhat higher. Heim (122) found variations from 1.38 to 4.57 per cent in lymph, while Arnold and Mendel (13) obtained a value of 3.56 per cent. The total protein concentrations determined by Gilligan, Volk and Blumgart (98) varied from 0.25 gram per 100 grams of water in subcutaneous edema fluid to 4.36 grams in a case of ascites secondary to carcinoma. Their average value for all fluid proteins (chest, ascitic and edema fluids) was 1.49 grams per 100 grams of water.

The globulin fraction in cattle fluid, as determined by the method of Butler and Montgomery (46), averages 0.18 gram per 100 grams of water, with an average albumin content of 0.71 gram. The average albumin-globulin ratio of the fluid is 3.9, in contrast to an average ratio of 1.1 in the serum. The globulin concentration and the albumin-globulin ratio vary more in the fluid than in the serum, both in normal (222) and pathological states (52, 221). Similarly, marked variations in the albumin-globulin ratio in other body fluids were found by Gilligan, Volk and Blumgart (98). They may be due to difficulties in fractionation of the protein constituents when the total protein content is low, but more likely represent variations in capillary permeability.

The presence of serum proteins in lymph and other body fluids has

never been explained adequately. The majority of investigators (55, 63, 72, 79, 156, 274) have concluded that it is the result of slight capillary permeability to protein and subsequent concentration. Other workers (162, 163, 174, 257) consider that capillary permeability to proteins is negligible. Formation of the proteins in situ is another possibility but has never been investigated. The available evidence indicates that normal capillary walls are slightly permeable to proteins, but whether sufficiently so to explain the concentration of protein in body fluids is unknown.

The low globulin concentration and high albumin-globulin ratio of the fluid compared to those of the serum are in accord with the results obtained for other body fluids (lymph, edema fluid and ascitic fluid) (73, 80, 100, 277, 279). If one assumes that the serum proteins enter the fluids through the capillary walls, the high albumin-globulin ratio indicates greater permeability to albumin than to globulin, corresponding with the difference in molecular weights of albumin and globulin. A similar difference is found in their rates of removal from joints (20), which will be discussed below.

In addition to albumin and globulin, synovial fluid contains mucin. It is this mucoprotein that is responsible for the high viscosity and presumably for the lubricating value and high colloidal osmotic pressure of the fluid. The concentration of mucin varies markedly in different joints. The average concentration in the astragalotibial joints of cattle is 0.14 gram per 100 cc., and in the carpometacarpal joints, 0.60 gram. Fisher (83) found 1.95 per cent mucin in normal human fluid and only 0.13 per cent in fluid from oxen. Cajori and Pemberton (52) reported a mucin content of 0.42 per cent in fluid from a patient with generalized edema. Achard and Piettre (1) found 5.7 per cent mucin in normal human fluids. We cannot account for the latter value since the maximal total protein concentration of normal fluid, including mucin, is 3.23 (222). The average mucin concentration in normal human fluids obtained in this laboratory is 0.85 gram per 100 cc., with variations from 0.55 to 1.10 grams.

The exact chemical composition of synovial fluid mucin has not been established. It has been generally accepted that it is a glycoprotein, and not a nucleoprotein as originally suggested by Hammarsten (112). Various elementary analyses have been reported (3, 128, 219, 230). Of greater importance than such analyses is the determination of the structural composition and the nature of the component protein and polysaccharide. The identity of the protein has not been

established but it probably is not a single globulin, as suggested by Meyer, Smyth and Dawson (181). The polysaccharide contains equimolar parts of acetylglucosamine and a hexuronic acid (181). Meyer, Smyth and Dawson (181) have concluded that synovial fluid polysaccharide is identical with the polysaccharides of vitreous humor, umbilical cord and group A hemolytic streptococcus. The results of enzymatic studies in our laboratory also suggest the identity of the mucins and polysaccharides of synovial fluid, vitreous humor and umbilical cord, but proof will not be possible until the nature of the proteins and polysaccharides has been established with certainty. It is significant that mucin obtained from subcutaneous tissue is also similar, physically and enzymatically, to the above mucins (218). While the majority of investigators have considered the mucin of joint fluid to be a glycoprotein, Meyer, Smyth and Dawson (181) conclude that the polysaccharide acid occurs in the fluid either free or united to protein in salt linkage only. Such an hypothesis is not in accord, however, with all of the physical and chemical properties of synovial fluid mucin.

Normal synovial fluid contains no fibrinogen as suggested by its failure to clot. The absence of fibrinogen has been corroborated in this laboratory by precipitation experiments with 1.1 M phosphate solutions at pH 6.5.

Distribution of Non-electrolytes. The average concentration of urea in cattle fluid per 100 grams of water (8.2 mgm.) is approximately the same as that of the serum (8.5 mgm.), as would be expected if serum and fluid were separated by a membrane permeable to this substance. The distribution ratios of total non-protein nitrogen (0.87) and uric acid (0.84) between fluid and serum in cattle are somewhat lower than that of urea, but of the same general magnitude. These findings are in accord with those reported by Hare and Cohen (115) for normal horse synovial fluid, and by other workers for pathological fluids (8, 51, 52, 62, 191, 221). The distribution ratio for nonprotein nitrogen in normal human fluid is 0.91 (222).

Although the average distribution ratios for urea, uric acid and nonprotein nitrogen are slightly below 1.00, in individual cases equal concentrations in fluid and serum have been observed. It is evident, therefore, that these non-electrolytes are completely diffusible through the membrane separating serum and fluid.

The average concentration of sugar in cattle fluid is, on the other hand, considerably lower in the fluid than in the serum, and the in-

dividual variations are much greater than those for other substances. These differences may be due in part to the fact that the animals were not fasting. Another explanation may be that the ante mortem struggle caused a sudden rise in blood sugar which was not reflected in the joint fluid by the time of death. Analysis of human fluids (222) shows that one cannot ascribe these variations to the presence of a non-diffusible portion of glucose in the serum as suggested by Brull (43). In most cases, the distribution ratios of sugar between fluid and serum are approximately 1.00. Complete diffusion of reducing substances has been found also in studies on plasma and glomerular urine (273).

Cholesterol and fatty acids are not found in normal fluid. This is in accord with the generally accepted theory that the capillary membrane under normal conditions is not permeable to these substances.

In summary, the distribution of non-electrolytes is consistent with that found in a dialysate of blood plasma.

Enzymes. The enzymes of normal synovial fluid have not been studied in detail. Podkaminsky (206) found that diastase, lipase and protease are present, but did not detect any catalase. The fluid of cattle has been found to have a higher average phosphatase activity than the serum. Greater variations are encountered in the fluids than in the sera. Further studies are needed in order to explain such variations and to determine the rôle of enzymatic activity in the metabolism of articular structures.

Hydrogen Ion Concentration. The average pH of cattle fluid is 7.31 as compared with an average pH of 7.42 for the serum. Few reports of the pH of normal human synovial fluid have been made. Horiye (129) found post mortem fluid to be weakly alkaline to litmus. Seeliger (241) reported its pH as 8.2 to 8.4. Boots and Cullen (38) found a pH of 7.34 in fluid from a patient with generalized edema. Pescatori (203) studied forty post mortem fluids and found an average pH of 7.95 with variations from 7.53 to 8.02. The average pH of normal human fluids obtained post mortem, as determined in this laboratory, is 7.40.

Distribution of Electrolytes. The concentrations of chloride and bicarbonate are higher in the fluid than in the serum of cattle, while the concentrations of sodium, potassium, calcium and magnesium are lower in the fluid than in the serum. The concentration of total inorganic phosphate is practically the same in fluid and serum. These distributions are, in general, such as would be expected from the laws regulating membrane equilibrium. The close agreement of the ratios

with those for edema and ascitic fluids, lymph and the "in vivo dialysate," and with the theoretical Donnan distribution ratio is apparent from table 2. The acid-base equilibrium between serum and fluid is presented graphically (see fig. 1).

The excess of chloride in the fluid bears about the same relation to the excess of protein in the serum as has been found when lymph,

TABLE 2

Comparison of the distribution ratios for synovial fluid (220), edema fluid (98, 101, 120, 168), the "in vivo dialysate" (106), ascitic fluid (67, 105, 120, 190) and lymph (13, 122) and the theoretical Donnan ratios

	SYNOVIAL FLUID	EDEMA FLUID	"IN VIVO DIALYSATE"	ASCITIC FLUID	LYMPH
$\frac{Cl_s}{Cl_f}$	0.99	0.97	0.98	0.98	0.95
$\frac{HCO_{3s}}{HCO_{3f}}$	0.94	0.96	0.97	0.97	
$\frac{PO_{4s}}{PO_{4f}}$	1.00	1.03	1.17	1.12	1.07
$\frac{Na_f}{Na_s}$	0.93	0.97	0.91	0.94	
$\frac{K_f}{K_s}$	0.75	0.73	0.78	0.94	
$\sqrt{\frac{Ca_f}{Ca_s}}$	0.83	0.80	0.76	0.84	
$\sqrt{\frac{Mg_f}{Mg_s}}$	0.88		0.66	0.86	
Theoretical Donnan ratio	0.93	0.96	0.93	0.97	

edema fluids and the "in vivo dialysate" are compared with serum. The excess of chloride in the fluid in proportion to the excess of protein in the serum, however, is slightly lower than that found for the other fluids (97). This difference may be related to the nature and relative concentration of the proteins in synovial fluid. The high-albumin-globulin ratio in the fluid would tend to increase the base-binding

power per gram of total protein. The presence of mucin may further augment the base-binding power, as indicated by the results of solubility experiments on mucin (265).

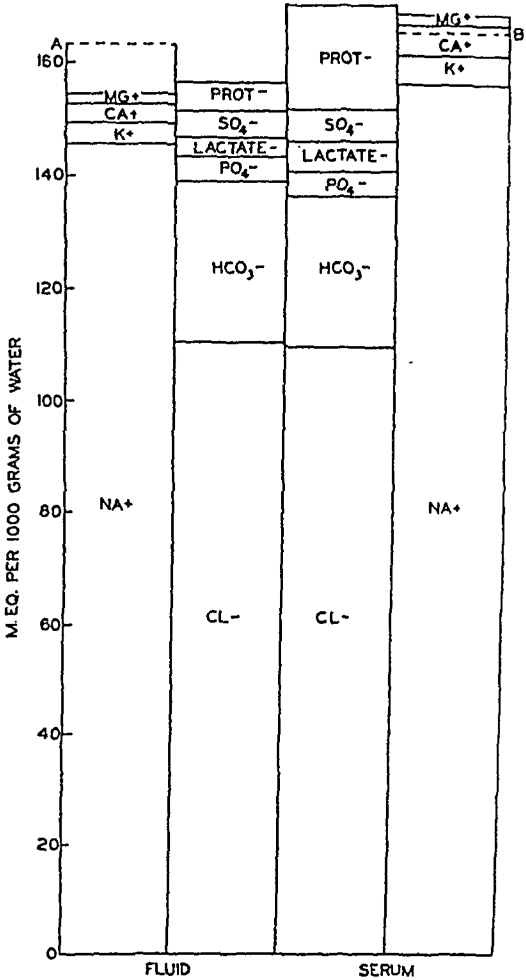

Fig. 1. The distribution of anions and cations between serum and normal cattle synovial fluid.

It will be noted that the summations of individual bases in the fluid and serum do not correspond exactly with the average determined total base values for fluid (A) and serum (B).

The formulae of Van Slyke, Hastings, Hiller and Sendroy (268) were used in estimating the proteinate. In the case of mucin, the base-binding power was assumed to be ten times the average base-binding power of albumin and globulin.

The average distribution ratio of the total inorganic phosphate is 1.00, essentially the same as that of chloride. The similarity of these ratios indicates that inorganic phosphate is entirely diffusible, and that its distribution is determined by the same laws of membrane equilibrium as regulate the distribution of chloride between serum and synovial fluid. This result is in accord with the findings of Brull (43), Heim (122) and Walker (271, 272), but in contrast to the conclusions of Greene and Power (106) and Gilligan, Volk and Blumgart (98).

The average ratio $\frac{(HCO_3)\ s}{(HCO_3)\ f}$ is 0.94, which is in close agreement with the theoretical Donnan ratio, and conforms fairly well with the bicarbonate ratio found for other fluids. Deviation from the chloride ratio may depend on several factors. The bicarbonate ratio represents that between arterial blood and fluid, and, as would be expected, this ratio has been found to be lower than that between venous blood and fluid. Furthermore, the discrepancy may be due to a difference between the carbon dioxide content of blood from the carotid artery and that of blood from capillaries around the knee. In addition, true equilibrium probably never exists because carbon dioxide is constantly being poured into the fluids from the tissues to be removed by the blood (204).

The average ratio $\frac{\text{(lactic acid) s}}{\text{(lactic acid) f}}$ is 2.11. The concentrations in individual cases show marked variations. The extremely high distribution ratio and the variations in concentrations in individual sera and fluids are presumably explicable as in the case of the sugar by the fact that the ante mortem struggle caused a sudden rise in the blood lactic acid which was not reflected in the joint fluid by the time of death.

The average ratio $\sqrt{\frac{(SO)_4\ s}{(SO)_4\ f}}$ is 1.06, 7 per cent higher than the chloride ratio. Since the determination of sulphate in blood and fluid is not exact, the 7 per cent deviation is not of great significance, and the sulphate ratio may be considered in general agreement with the chloride ratio.

The average ratio $\frac{\text{(total anions) s}}{\text{(total anions) f}}$ is 0.99, which agrees well with the ratio found for the "in vivo dialysate" (106), and with the chloride ratio.

The average ratio $\frac{(\text{Na})\,\text{f}}{(\text{Na})\,\text{s}}$ is 0.93, which is identical with the theoretical Donnan ratio, but slightly lower than the chloride ratio. It is in fairly good agreement with the ratios found by other workers. The slight deviation from the chloride ratio may indicate that a small percentage (6 per cent) of the sodium is held in the serum in a non-diffusible form, presumably bound to protein.

The average ratios $\frac{(\text{K})\,\text{f}}{(\text{K})\,\text{s}}$ (0.76) and $\sqrt{\frac{(\text{Mg})\,\text{f}}{(\text{Mg})\,\text{s}}}$ (0.88) were obtained from a smaller number of analyses, the results of which varied considerably. However, the deviation from the chloride ratio is great and of the same magnitude as that found for other fluids. One can conclude that part of the potassium (approximately 25 per cent) and part of the magnesium (approximately 30 per cent), as well as part of the calcium, are held in the serum in a non-diffusible state.

The average ratio $\frac{(\text{total base})\,\text{f}}{(\text{total base})\,\text{s}}$ (0.98) is identical with the chloride ratio. The results of the individual determinations of the total base concentration, however, varied markedly. The distribution ratio of total base concentrations obtained by summation of the average concentrations of the individual cations in the fluid and serum is 0.91. This value may be a more accurate indication of the base held in the serum in a non-diffusible state.

The average ratio $\sqrt{\frac{(\text{Ca})\,\text{f}}{(\text{Ca})\,\text{s}}}$ is 0.83, and indicates, as do the similar calcium ratios obtained for the "in vivo dialysate" and other fluids, that part of the calcium is held in the serum, presumably bound to protein. Of more significance than the distribution ratio of total calcium is that of ionized calcium. Calculation of the calcium ion in serum and fluid from the protein and total calcium concentrations (McLean and Hastings, 178) gives a ratio $\frac{(\text{Ca}^{++})\,\text{f}}{(\text{Ca}^{++})\,\text{s}}$ of 1.18. This ratio is much higher than would be expected from the laws of membrane equilibrium. The difference may be explicable in part by the fact that, in calculating the calcium ion concentrations of the serum and fluid, no consideration was given to the difference in pH and albumin-globulin ratios, but in larger part by the fact that the mucoprotein was included as part of the total protein and assumed to have the same effect as the serum proteins. That this is incorrect is evident from a comparison of the calcium concentration of synovial fluid with that of other body

fluids known to be dialysates of blood plasma. A review of the results reported on such fluids gives an average empirical ratio $\frac{(\text{Ca}) \text{ dialysate}}{(\text{Ca}^{++}) \text{ serum}}$ of 1.33 (Hastings, 119). Using the average calcium ion concentration in the cattle serum (1.21 mM. per kgm. of water), the calcium concentration of synovial fluid calculated from the above empirical formula is 1.61 mM. per kgm. of water in contrast to the observed value of 1.90 mM. The difference (0.29 mM. per kgm. of water) represents an estimate of the calcium bound by mucin. In terms of millimols of calcium bound per gram of mucin the figure is 0.23 mM., a value approximately ten times that obtained for serum proteins (178). This is in accord with the results of experiments on mucin discussed above (265), which indicate that the base-combining power of mucin is high.

The distribution of electrolytes agrees, in general, with that expected from the Gibbs-Donnan theory of membrane equilibrium, and with the results obtained by Greene and Power (106) in the study of the "in vivo dialysate," and by various workers in the study of other fluids which have the composition of dialysates.

Theories of Origin. Many theories concerning the origin of synovial fluid have been proposed and will be presented in brief.

1. Synovial fluid is a product of glandular synovial membrane cells. This concept originated with Havers (121) and was supported by many subsequent workers (14, 32, 42, 64, 65, 102, 135, 149, 165, 179, 208, 236, 238, 252, 263). It was based chiefly on physiological deduction and gross examination. Drawings or photomicrographs of glandular cells have never been presented.

2. Synovial fluid is a mixture of the products of disintegration of synovial membrane and a transudate from the capillaries and lymphatics. This theory, presented by Frerichs in 1846 (91), has been supported in a modified form by other workers (4, 54, 78, 83, 110, 111, 118, 125, 169, 206, 210, 258). Histological studies served as the chief basis for such conclusions.

3. Synovial fluid is formed from the products of attrition of cartilage. Originally proposed by Ogston (197) and Banchi (18), this theory has received no support except for a statement by Fisher (83), in which he suggested that a portion of the synovial fluid mucin might be derived from articular cartilage as it becomes worn.

4. Synovial fluid consists of substances elaborated by synovial membrane cells with the addition of a transudate from the capillaries

and lymphatics (89, 151, 176, 229). This theory is for the most part based on histological studies with special stains.

5. Synovial fluid is the liquid matrix of the connective tissue lining an enlarged tissue space, the joint cavity (30, 62, 132, 144, 147). According to this theory, which is based on analogy suggested by embryological and histological studies (130), the synovial fluid mucin corresponds to the mucoid constituent of other connective tissues. According to Vaubel (270) mucin is the ground substance of synovial tissue.

6. Synovial fluid is a dialysate from the blood capillaries. This theory was first suggested by Bichat (34) (1812) who proved that the "glands" described by Havers were fat deposits and stated that synovial fluid is formed by "exhalation" of the blood capillaries. This concept has been supported by subsequent workers (8, 22, 35, 52, 71, 92, 124, 141). More recent reviews (204, 235) of the existing data on synovial fluid and the experimental evidence from this laboratory presented above have led to the conclusion that synovial fluid is in ready diffusion equilibrium with plasma. Except for the presence of mucin, albumin and globulin, it could, therefore, be considered a diffusate or a simple ultrafiltrate of serum.

The concept that synovial fluid is a dialysate of blood plasma to which mucin is added as the fluid diffuses through the connective tissue surrounding the joint is not fundamentally different from the theory that synovial fluid represents the liquid matrix of connective tissue, nor does it differ materially from that in which synovial fluid is considered a combination of synovial membrane cell products and a transudate from the capillaries.

The distribution of electrolytes and non-electrolytes between serum and normal synovial fluid is in accord with the concept that normal synovial fluid is a dialysate in equilibrium with blood plasma. This relationship is also suggested by the marked vascularity of the synovial intima whose numerous capillaries and venules are in close proximity to the articular lumen. Such a theory explains all known facts of the physical and chemical composition of synovial fluid except the presence of albumin, globulin and mucin. The presence of albumin and globulin can be ascribed presumably to slight capillary permeability to protein as discussed above. The presence of the mucin, whether it be formed by the surrounding connective tissue, as seems most likely, or by cartilage, in no way invalidates the theory.

Little is known concerning the source of synovial fluid mucin. Kling

(149) considers its presence, as determined by the "sac phenomenon," in both synovia and saliva as evidence of the secretory nature of synovial fluid. Kling failed to appreciate that the formation of a sac in acetic acid merely indicates the presence of a precipitable quantity of mucin and does not prove the secretory origin of synovial fluid. We have never observed glandular cells in synovial tissue and are not aware that microscopic evidence of such cells has been presented. In fact, histological studies as described above show that synovial membrane consists of modified connective tissue. The theory that mucin is formed in cartilage is supported only by a similarity of staining reactions (18). No chemical identity between mucin and any constituent of cartilage has been established. Other workers have described "granules" which have been interpreted as representing mucin in synovial membrane cells (54, 88, 149, 176, 229), but the staining methods employed are open to question. There is good evidence to suggest that synovial fluid mucin originates as the mucinous component of the connective tissue lining the joint (30, 62, 132, 140, 144, 147, 270). King (147) has offered the demonstration of Golgi apparatus in support of this theory. Extraction from the subcutaneous tissue of rabbits and the tissue lining the astragalotibial joints of cattle of a substance similar to synovial fluid mucin as shown by its physical properties and by enzymatic studies (218) confirms the suggestion that mucin is formed by the connective tissue cells surrounding the joint. Its entrance into the joint is made possible by the diffusion of plasma water from the underlying vessels through the subsynovial tissue and membrane.

The high colloidal osmotic pressure and high calcium concentration of the synovial fluid form the only essential differences between the synovia and other fluids with the composition of plasma dialysates. These properties of joint fluid are presumably due to the presence of mucin and indicate that mucin plays a rôle in the exchange of water and other substances between the vascular system and the joint cavity. The finding of similar, if not identical, mucins in subcutaneous tissue and synovial membrane suggests that mucin may have a similar action in all connective tissue fluid.

The functions of synovial fluid are partly those inherent in its position as intercellular connective tissue fluid and partly those associated with the specialized functions of joints. It is the main source of nourishment for the avascular articular cartilage and aids also in the nutrition of the superficial cells of the synovial membrane, particularly in villi

without blood supply. Bordier (39) suggests that it may act as an important cohesive force in the joint. Since Havers (121), the significance of synovial fluid as a lubricant has been generally acknowledged. Jones (139) devised an experiment to test articular lubrication. He used the proximal interphalangeal joint, the middle phalanx remaining fixed. Applying the Osborne-Reynolds law, and comparing load and friction, Jones concluded that fluid film lubrication is the usual form in human joints, but that some solid friction must occur when speed and/or eccentricity are insufficient to maintain a pressure film. The fluid film formed withstood a load of 900 pounds per square inch, a load that will crush bone.

Exchange of substances through the articular membrane. For obvious reasons the interest of workers has centered on the mechanism of exchange between the joint cavity and the body as a whole. First, because maintenance of the normal state and function of the joint requires entrance of nutritive material and removal of potentially toxic end products. Next, because the anatomical structure of the synovialis differs radically from that of the other body membranes across which transfer has been studied, as peritoneum, pleura and vascular endothelium. Finally, because in joint diseases, it frequently appears to be this phase of the physiology which is disrupted, early and to a striking degree.

Histological studies, as reviewed above, show that the joint cavity should be considered a large tissue space. The connective tissue enclosing it, although constituting an anatomical boundary, is not a true membrane such as the peritoneum, pleura and those of the choroid plexus and glomerulus. The contents of the articular cavity are presumably in direct communication with the matrix of the synovial tissues. Therefore, one can assume that exchange of substances between the vascular or lymphatic system and the synovial fluid involves the same processes as those governing the exchange in any connective tissue fluid. Such transfer necessitates passage through an endothelial wall as well as diffusion through the intercellular spaces of the synovial membrane. In the case of synovial membrane, the term, permeability, implies these two processes and will be used in this sense throughout the following presentation. Such permeability will be regulated by various physicochemical factors. It will depend on the capillary and lymphatic permeability, the charges of the diffusing substances and any oxidation-reduction potentials involved. As was pointed out above, it will also be subject to the laws of equilibrium

across a semipermeable membrane as formulated by the Gibbs-Donnan theory. The concentration of synovial fluid constituents will vary with their concentration in the plasma and will be influenced by the metabolic activity in the joint cavity.

Comparison of the composition of joint fluid with that of plasma indicates that non-electrolytes diffuse readily in either direction between blood and synovial fluid and that electrolytes are distributed in accord with the Gibbs-Donnan theory. Direct evidence of the mode of transfer across the synovial membrane has been obtained by studying the disposition of various substances following intravenous and intra-articular injection.

Thiocyanate and sugar diffuse readily into the joint spaces following intravenous injection in calves (285). The appearance time in the fluid varies; thiocyanate was detected ten minutes after injection, whereas the glucose concentration showed no rise for twenty minutes. A similar twenty-minute lag was apparent when the blood sugar was falling. The difference between thiocyanate and sugar is significant since both substances are of small size and freely diffusible and it emphasizes the multiplicity of factors involved in transfer.

Thiocyanate diffusion equilibrium between joint fluid and serum was attained in from one to five hours. In the experiments of Lavietes, Bourdillon and Klinghoffer (166), equilibrium was reached in one-half to one hour, but it was determined by stable blood concentrations rather than by true diffusion equilibrium. In both series of experiments the concentration of thiocyanate in the fluids averaged approximately 9 per cent lower than that of the blood, indicating that part of the thiocyanate is held in the blood in a non-diffusible state.

The concentration of thiocyanate in the aqueous humor was much lower than that in the synovial fluid, and only traces were present in the cistern fluid. The differences indicate anatomical and physiological variations in the barriers and are similar to those found in protein studies discussed below.

Readily diffusible substances of small molecular dimensions in homogeneous solution are removed from joints primarily by way of the subsynovial blood capillaries, as shown by the investigations of Rhinelander, Bennett and Bauer (216). In their experiments, absorption of mecholyl after intra-articular injection was measured either directly by its effect on the blood pressure, or indirectly by biological assay of the joint washings. Mecholyl in aqueous solution was readily absorbed from all joints. The effect on blood pressure was evident

within thirty to sixty seconds after injection. Passive exercise increased strikingly the rate of absorption, presumably by raising the intra-articular pressure and augmenting the flow of blood. Absorption was slightly increased from mildly inflamed joints but was definitely greater from those in which severe, acute inflammation had been produced. This was true of both exercised and resting joints. Intra-articular injection of adrenalin completely prevented absorption of mecholyl, presumably by constricting the subsynovial capillaries. Aqueous solutions of pituitrin and pilocarpine also were readily removed from joints by way of the blood stream. Determinations on thoracic duct lymph showed that little if any mecholyl was absorbed by way of the subsynovial lymphatics.

Intra-articular injection of dyes has given further evidence of the mode of absorption of diffusible substances of relatively small size. The uniform result of these experiments, as first formulated by Braun (40) is that such substances pass rapidly into the intercellular spaces of the membrane by diffuse penetration to variable depths (83, 260). The subsequent route of removal, however, is debated. Tillmanns (260) described the collection of dye in the venules of the synovial membrane. Although Braun (40), Tillmanns (260), Rynearson (228) and Fisher (83) found varying amounts in the lymphatics, the consensus of opinion (83, 158, 228, 260) is that diffusible dyes are absorbed largely by capillaries and only slightly by lymphatics. Allen (6), on the other hand, concluded that removal of diffusible dyes takes place entirely by way of the lymphatics. Since his experiments were performed on dead animals perfused with saline, the conclusions are not valid.

Experiments in which potassium or sodium iodide has been determined in urine or saliva at various intervals after intra-articular injection (53, 83, 199) are less indicative of the mode or rate of absorption because these salts cause marked inflammation of the synovial membrane and are readily stored in the organism. The amount of phenolsulphonephthalein remaining in the knee joints of normal dogs at the end of an hour has been determined (50). While it is impossible to draw conclusions from these experiments as to the mode of absorption, they afford a basis of comparison for similar studies in joint diseases.

The rapid rate of diffusion of salts from the joint cavity and from the capillaries into the synovial membrane is apparent from the experiments of Mayeda (176). After injecting sodium salicylate intravenously in rabbits, he introduced an iron salt into the joint cavity

and could demonstrate ferric oxide salicylate in the membrane ten minutes after injection.

Permeability of the synovial membrane to substances of large molecular size, such as proteins, is of special significance in the physiology of joints because of their effect on osmotic pressure, and thereby on water exchange. The entrance and removal of colloids involves a more complex mechanism than the exchange of readily diffusible small molecules. The presence of albumin and globulin in normal synovial fluid indicates presumably that the capillaries are slightly permeable to proteins. The high albumin-globulin ratio in the fluid suggests a greater permeability to albumin than to globulin in corroboration of the findings of other investigators in lymph, edema and ascitic fluid (73, 80, 100, 277, 279). Experimental studies in this laboratory (27) have given further knowledge of the permeability of the synovial membrane to proteins. Egg albumin was found in the knee joint of rabbits within five minutes after intravenous injection. The foreign protein concentration in the joint fluids tended to vary directly with the concentration in the serum. Differences were encountered, however, between individual animals and occasionally between corresponding joints of the same animal. Such individual variations represent local, and presumably temporary, changes in the permeability of the membrane. The appearance time of larger molecules, horse serum albumin and euglobulin was approximately twenty minutes longer than that of egg albumin. In experiments of twenty-four hours' duration or longer, the concentration in the joints tended to increase, indicating that the rate of removal is slower than the rate of entrance. The fact that the globulin concentration increased sooner than that of albumin suggests that globulin is removed more slowly.

Examination of other body fluids in the above experiments showed marked differences in the ease with which the foreign proteins passed from the vascular system into synovial fluid, aqueous humor, spinal fluid and urine. The proteins appeared in aqueous humor at approximately the same time as in synovial fluid, but were present in lower concentrations in the former. In the experiments of longer duration there was no tendency to accumulation of the proteins in the aqueous humor, suggesting that the rate of removal approached the rate of entrance. In spinal fluid the proteins appeared only rarely and in minimal amounts, indicating that the choroid plexus is less permeable to these substances. Egg albumin was eliminated readily by the kidney. The horse serum proteins appeared only occasionally in the

urine, and their concentrations in the blood remained relatively high for several days. The variation in permeability between the different membranes was shown to be dependent both on the molecular size of the proteins and on the anatomical and physiological character of the intervening barriers. With the exception of the kidney glomeruli in the case of egg albumin, the synovial membrane is the most permeable. This greater permeability and the slow rate of removal probably explain the higher total protein content of normal synovial fluid in comparison with aqueous humor and spinal fluid.

Proteins, in contrast to small, readily diffusible substances, are removed from joints only by way of the lymphatics. This mode of removal of colloids was first suggested by Böhm (37) who noted fat globules in the inguinal lymph nodes twenty-four hours after injection of milk into the knee joint. Subsequently, lymphatics were outlined after intra-articular injections of colored mucilage (83), a colloidal suspension of silver (83) and a mixture of turpentine, dye and ether (246). Conclusive evidence that proteins are removed only by the lymphatics was obtained in the investigations of Bauer, Short and Bennett (20). Egg albumin and horse serum albumin were found in thoracic duct lymph thirty minutes after injection into the knee joints of dogs if the legs were passively exercised or massaged. None of the injected protein appeared in the blood stream when all communications between the lymphatic and vascular systems were eliminated. Horse serum globulin was removed from joints with difficulty, if at all, even when the leg was exercised.

The removal of particulate matter such as carbon particles, bacteria and blood cells is achieved by a combination of several distinct processes. It takes place more slowly and is less complete than the removal of soluble substances. The earliest experiment, that of Böhm with cinnabar (37), was inconclusive but von Mosengeil (183) detected India ink in all lymphatics between knee joint and inguinal nodes two minutes after intra-articular injection and joint massage. The rapidity of spread recorded here is difficult to reconcile with the results of subsequent workers, unless one assumes that the membrane ruptured or leakage occurred at the site of puncture. Braun (40), on the basis of careful histological study, described the steps in the removal of the carbon particles of India ink. The increased intra-articular pressure caused a large part of the material to enter directly into the intercellular spaces of the membrane. Another part was removed by connective tissue phagocytes and to a smaller extent by polymorphonuclear

leukocytes emigrating from the membrane and carrying the substance back into it. A third part remained in the joint cavity to be ultimately organized. A small portion was taken up by the synovial cells bordering directly on the joint space. From all of these sources, by repeated phagocytosis, some carbon finally reached the regional and distant lymphatics. More recent investigations (143, 157, 159, 176, 228, 247), some of which were done without knowledge of Braun's work, have led to strikingly similar conclusions. Mayeda (176) pointed out that the intensity of resorption differs in the various parts of the membrane, according to anatomical structure. In Key's outstanding report (143), the wide ultimate distribution of carbon in bone-marrow, liver and spleen is described.

The value of joint exercise, particularly passive, and of massage for increasing absorption from joints has been demonstrated repeatedly (6, 20, 40, 157, 183, 187, 189). It is probable that the increase is achieved mainly by augmenting the flow of blood and lymph and not merely by elevating the intra-articular pressure (251, 260). The effect of compression of the joint on absorption has not been conclusively established. Tillmanns (260) reported increased absorption of a water-soluble dye. Kroh (157) and Müller (189) concluded that compression made no appreciable difference in resorption of particulate matter. The results do no more than suggest that compression increases absorption by way of the capillaries and does not affect removal by the lymphatics.

The entrance of particulate matter into the joint has been less thoroughly investigated. Kuhns and Weatherford (159) found that carbon particles injected into the abdominal wall of rats appeared in the histiocytes of the synovial membrane, but in smaller quantities than in the liver, spleen, kidney and bone marrow. Experimental studies (61, 68, 69, 180, 223, 244) have shown that living bacteria gain access more readily to synovial fluid than to spinal fluid, aqueous humor and urine. The relatively greater permeability of the synovial tissue as compared with true membranes is again apparent.

Alterations in physiology produced by disease. While a consideration of articular disorders and of the anatomical changes produced thereby is not within the scope of this discussion, certain features of pathological joint physiology serve as an adjunct to our understanding of normal joint function. The changes produced by disease depend on two fundamental alterations in function: altered permeability of synovial tissue and disturbance of intra-articular metabolism.

The former permits increased entrance of water, readily diffusible substances, proteins including fibrinogen, leukocytes, antibodies and presumably enzymes. The distribution of diffusible substances follows the laws that apply to normal joints. In the case of a utilizable material, such as sugar, equilibrium is established, but with increased consumption, as in the case of septic joints, the fluid sugar is markedly decreased (8, 52, 62, 141, 191, 221). The low sugar content in some cases of rheumatoid arthritis, on the other hand, is due, at least in part, to decreased permeability of the membrane to sugar (266). Transfer of thiocyanate into these same joints is essentially normal and entrance of proteins is increased. The mechanism of this differential permeability is not understood, but the experimental results emphasize again that a variety of factors is involved in the transfer. The amount of protein entering the joint space varies directly with the degree of inflammation. It is slightly greater than normal in traumatic joints and markedly increased in severe rheumatoid and septic arthritis (221). The proportion of globulin in the entering protein increases as the membrane becomes more permeable, and therefore the albumin-globulin ratio more closely approaches that of serum.

Alterations in the membrane lead also to diminished removal of colloidal and particulate matter. Experimental evidence suggests that this is due to a decreased number of synovial lymphatics (158, 201). The reduced absorption, combined with greater entrance, may account for the increasing protein concentration and decreasing albumin-globulin ratio in rheumatoid effusions of long duration (221). The abnormally high concentration of colloidal material raises the osmotic pressure and results in persistence of the effusion.

The altered metabolism of the joint is most apparent in the case of sugar and mucin. Greater utilization of sugar results from various factors: increased cellular activity of fixed tissue cells, a larger number of leukocytes, an enhanced enzymatic activity and bacteria, if present. Greater metabolic requirement for sugar, coupled with decreased supply, may lower the fluid sugar to a level insufficient for adequate nutrition of cartilage (266). In the case of mucin both formation and destruction are affected. In traumatic effusions the unit concentration of mucin remains normal despite a great increase in fluid volume (221). Whether the inflammation produced by trauma stimulates formation of mucin by the connective tissue cells or merely increases the amount of mucin carried into the joint has not been established.

Infectious fluids show a decreased concentration of mucin, a reduced viscosity and an atypical precipitate with acetic acid. The similarity of these changes to those produced by the bacterial enzyme, mucinase (218), suggests that increased destruction of mucin takes place rather than decreased formation. Such fluids also show an increase in the normal difference between the total glucosamine and the glucosamine of the precipitated mucin, again indicating accelerated breakdown of mucin.

JOINTS AS STRUCTURAL AND FUNCTIONAL UNITS. Finally a synthesis of the evidence will be attempted by integrating the individual results into a working scheme. We may thereby test their consistency and become aware of missing links.

Articulations serve the rather primitive purposes of motion and weight-bearing. Their structure, accordingly, is simple and firm. Cartilage in terms of comparative zoölogy is the oldest tissue in the human body (169) and all principal joint components have a common mesenchymal origin. The articular capsule, which consists of tough, fibrous and modified connective tissue, bounds with the epiphyseal cartilages a potential space partly filled with a hypocellular fluid of high viscosity. Articular construction allows ease of motion to be combined with relatively great stability. The viscous synovia aids by its cohesive action in uniting the articular ends and forms a strong fluid film upon which the cartilaginous surfaces glide with negligible *friction. The capsule and tendons' inserting into it, the ligaments* and muscular tone, impart steadiness to the joint, while cartilage by virtue of its remarkable elasticity is able to buffer the impacts to which the rigid skeletal system is exposed. Cartilage under normal conditions and particularly with advancing age is subject to wear and tear in a measure unequalled by any other tissue, with the exception of the integument. The resistance thus required excludes fragile and sensitive structures from the architecture of this tissue and may explain the disappearance of vascular and nervous elements at the time of birth. Hence the usual avenues of metabolic exchange are not available for articular cartilage and its subsistence depends largely, if not altogether, on the synovial fluid. We can merely suggest the manner in which metabolites enter and leave the cartilaginous substance, by pointing to its sponge-like resilience and the ability of dyes to diffuse along the intercellular system of fibrils. Since arterial blood does not come in contact with cartilaginous cells, the breakdown of glycogen must take place through anaerobic oxidation. This is confirmed by

the low respiratory quotient of this tissue. The histological picture characterized by a notable predominance of matrix over cells reflects both the physical requirements made upon cartilage and the disadvantageous metabolic conditions under which it functions.

Since the synovia conveys to cartilage its means of existence, the nature and origin of that fluid are of prime physiological importance. The distribution of electrolytes and non-electrolytes between blood and synovia and the marked vascularity of the synovial membrane indicate that the fluid is a dialysate of blood plasma. It contains an admixture of albumin, globulin and mucin. The presence of albumin and globulin is probably due to slight capillary permeability. The origin of mucin remains uncertain, but the evidence to date suggests that it is formed by the connective tissue cells of the synovialis and carried into the joint by the plasma dialysate as it diffuses through the synovial tissues. The factors influencing the formation of the synovial fluid require further investigation. Under normal conditions, the opposing forces of capillary pressure and osmotic pressure difference between plasma and fluid determine the volume of fluid present. Substances entering the joint from the systemic circulation must pass the capillary endothelium and diffuse through the interstitium of the synovialis. The concept of synovial tissue as a membrane analogous to the true body membranes should be discarded because uninterrupted continuity exists between the intercellular fluid of the synovialis and the synovia. Compared to true membranes, synovial tissue has a markedly greater permeability. Resorption of small molecules takes place almost entirely by the blood vascular system and only to a slight degree through the lymphatics, while the larger protein molecules are removed with difficulty and only by way of the lymphatics. The predominant cellular constituents of the synovial fluid are mononuclear phagocytes. They carry particulate matter and the cellular debris of wear and tear from the joint into the lymphatics in a manner which suggests that the synovial membrane shares in reticulo-endothelial activity. Balance of all the factors mentioned must obtain for the maintenance of a normal amount and composition of synovial fluid in the joint cavity. The slightly negative intra-articular pressure, varying with motion, would also appear to play a rôle in this equilibrium. The possible influence of the autonomic nervous system through its effect on blood vessels and cellular activity warrants further study.

Deviations from normal articular physiology, whatever their cause, consist chiefly of alterations in the synovial tissue and changes in

intra-articular metabolism. While the former leads to quantitative and qualitative disturbances of the exchange equilibrium, the latter results mainly in deficient supply of nutriment to cartilage. It may also reduce the available mucin to which synovia owes important physiological properties. Cartilage, once injured, shows little or no tendency to regenerate and repair takes place by invading fibrous tissue through marginal or subchondral proliferation.

The resemblance of the cytological characteristics of synovial fluid to those of tissue fluid, the similarity of its composition to that of other plasma dialysates, the presumptive identity of synovial fluid mucin with that obtained from subcutaneous tissue, lead to the conclusion that synovial fluid is an example of tissue fluid in general. Likewise, the embryological derivation of the joint cavity from a mesenchymal cleft, the histological and physiological properties of the synovialis, its great reparative and proliferative power and the nature of its cytological response to injury, all indicate that the articular lumen represents a connective tissue space.

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CARBON MONOXIDE ANOXEMIA

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Carbon monoxide derives its physiological interest from its property of combining with hemoglobin in a manner apparently identical with oxygen. The hemoglobin, however, has a very much greater affinity for carbon monoxide than for oxygen, and therefore carbon monoxide is a potent cause of anoxemia when it is mixed with the inspired air.

The combination of carbon monoxide with reduced hemoglobin, in the absence of oxygen, and the dissociation of carboxyhemoglobin, follow the same laws as govern the combination of oxygen with reduced hemoglobin and the dissociation of oxyhemoglobin. The curve relating the partial pressure of the gas to the percentage saturation of the hemoglobin is identical in shape for both gases, and its position is influenced by the same factors in both cases, i.e., pH, temperature, and salt content (Douglas, Haldane and Haldane, 1912). The difference lies in the range of partial pressures; while the hemoglobin of human blood becomes half saturated with oxygen at a partial pressure of about 30 mm., it is half saturated with carbon monoxide, under the same conditions of temperature, pH, etc., when exposed to a partial pressure of about 0.125 mm.

When blood is exposed to a mixture of O_2 and CO the Hb is divided between the two gases, the reaction obeying the law of mass action; i.e., the proportion of Hb combined with either gas depends upon the relative partial pressures of the O_2 and CO, and upon a constant which expresses the relative affinity of the blood for the two gases (Haldane and Smith, 1897)

$$\frac{[HbCO]}{[HbO_2]} = \frac{K[CO]}{[O_2]}$$

On the basis of this fact partition curves may be constructed for any blood for which the equilibrium constant (K) is known; these curves

relate the partial pressure of CO to the per cent saturation of the Hb with CO when the partial pressure of O_2 is kept constant. Similar curves can be constructed for O_2 when the partial pressure of CO is constant. Such curves are rectangular hyperbolas, provided the sum of the partial pressures of the two gases is sufficient to saturate the Hb completely. If this condition does not obtain, the curves show a curious "hump" in the area where the Hb is not completely saturated (Douglas, Haldane and Haldane, 1912). The rectangular hyperbola, that is, expresses only the reaction

$$HbO_2 + CO \rightleftharpoons HbCO + O_2$$

and when reduced Hb is present also the relationship is altered. But if the reduced Hb be ignored the hemoglobin that is combined still follows the same law and is divided between the oxygen and carbon monoxide according to the relative partial pressures of the two gases. The existence of this "hump" in the partition curves when reduced hemoglobin is present means that in the presence of low partial pressures of oxygen and carbon monoxide the addition of carbon monoxide will actually increase the proportion of oxyhemoglobin as well as that of carboxyhemoglobin. Haldane and Smith (1897) actually observed this effect; mice subjected to low oxygen tension seemed to improve when the carbon monoxide in the atmosphere was increased. At the time the accuracy of the observation was doubted, but it was realized later that it was a demonstration of the nature of the partition curve at these low partial pressures.

The value of the equilibrium constant (K) is practically independent of pH, salt content, laking, or purification of the hemoglobin (Haldane et al., 1912; Hartridge, 1912b; Roughton, 1934). Factors such as CO_2 tension, therefore, which markedly affect the oxygen or carbon monoxide dissociation curves of the blood, have no effect on the partition of hemoglobin between oxygen and carbon monoxide. This partition, however, is greatly affected by light, and also by temperature. The effect of light was observed by Haldane and Smith (1897) and has since been confirmed by numerous workers; Hartridge (1912) demonstrated that the most active part of the spectrum in this respect was the ultraviolet end, and concluded that it hastened the dissociation of HbCO in the presence of oxygen. The value of K varies inversely with the temperature at which equilibration occurs; Hartridge (1912) found a fall in the saturation of the hemoglobin with carbon monoxide of $\frac{1}{2}$ per cent for each 1°C. rise in temperature; Hartridge and Roughton (1923) found a temperature coefficient of 1.3 per 10°C.

Hartridge and Roughton in their work on the kinetics of hemoglobin have shown that a consideration of the velocity of the various reactions involved in the combination of hemoglobin with oxygen and carbon monoxide provides the explanation of many of the facts previously demonstrated experimentally (Hartridge and Roughton, 1923; Roughton, 1934). Light has been shown to accelerate the velocity of dissociation of HbCO. The great affinity of carbon monoxide for hemoglobin is due to the fact that HbCO dissociates very much more slowly than HbO_2, although oxygen combines with hemoglobin about 10 times as fast as does carbon monoxide under similar conditions of pH, temperature, and concentration of reagents.

The equilibrium constant (K) also varies considerably between animals of different species. Krogh (1909–1910) showed that the bloods of the rabbit and the ox differed markedly in their affinity for CO; his figures indicate a value of K = 288 for ox blood (37°C.) while for the bloods of different rabbits K varied from 115 to 205. The temperatures at which the different bloods were equilibrated varied, however, and when corrected to 37°C. the values of K for rabbit blood are approximately 83–140. Douglas and Haldane (1911) showed that the bloods of animals of different species exhibited marked differences in percentage saturation with carbon monoxide when equilibrated with the same air-carbon monoxide mixture, and similar differences were found between individuals of the same species. Burrell, Seibert and Robertson (1914) assessed the susceptibility of a number of small animals and birds to carbon monoxide by exposing the animals to atmospheres containing low concentrations of carbon monoxide and observing their symptoms. Barcroft and his co-workers (1924–25) determined the value of the equilibrium constant for the bloods of a number of vertebrate species, and found that at 15°C. it varied from K = 570 for a dog to K = 120 for a rabbit. They found considerable individual variation in mice, horses, and rabbits, but much less variation between human individuals. The value of K was correlated with certain spectroscopic properties of the blood; the distance, in Angström units, between the position of the α band of oxyhemoglobin and that of carboxyhemoglobin, defined as the "span," was shown to bear a definite relationship to the value of K in all the bloods examined. The curve relating these two properties was represented by the equation:

$$\log K = 0.05 \text{ A.U.}$$

where A.U. was the "span" in Angström units. The existence of individual differences among mice was confirmed by Killick (1937) who

relate the partial pressure of CO to the per cent saturation of the Hb with CO when the partial pressure of O_2 is kept constant. Similar curves can be constructed for O_2 when the partial pressure of CO is constant. Such curves are rectangular hyperbolas, provided the sum of the partial pressures of the two gases is sufficient to saturate the Hb completely. If this condition does not obtain, the curves show a curious "hump" in the area where the Hb is not completely saturated (Douglas, Haldane and Haldane, 1912). The rectangular hyperbola, that is, expresses only the reaction

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found values for K, determined at 37°C., ranging from 118 to 278. She also found individual differences in humans, and records values of K (at 37°C.) for 4 individuals of 233, 240, 246 and 272 (1936); the first three values lie within the limits of experimental error, but the fourth value, which was obtained repeatedly for that individual, indicates a difference outside these limits. Douglas, Haldane and Haldane (1912) record values for two individuals of 246 and 299 (at 37°C.). Sendroy, Liu and Van Slyke (1929), using a gasometric method to estimate the percentage saturation of the hemoglobin, found an average value for K of 210 in six individuals; the variation between these individuals was within the limits of experimental error. There seems little doubt that differences do exist between the hemoglobins of human individuals in this respect, but until more data are available it is impossible to say how wide the variation is. In most of these experiments the constant was determined by equilibration of whole blood with a series of air and carbon monoxide mixtures, in some diluted blood was used. The percentage saturation of the blood with carbon monoxide after equilibration was determined either by a gasometric method (Van Slyke and Robscheit-Robbins, 1927), by means of the reversion spectroscope (Hartridge, 1912a), or by the carmine method (Douglas and Haldane, 1912).

Effects of breathing carbon monoxide. The relationship between the partial pressures of oxygen and carbon monoxide and the saturation of the blood with carbon monoxide enables one to predict approximately the final blood saturation of a man who is breathing air containing a small amount of carbon monoxide, but it gives no information as to the speed with which that saturation will be reached. The rate at which the percentage saturation increases in different animals is proportional to the respiratory exchange per unit of body weight (Haldane, 1895b), and therefore in small animals absorption will be rapid and the final equilibrium will be reached much more rapidly than in man; the mouse reaches equilibrium with the mixture it is breathing twenty times as rapidly as man, and hence its use as an indicator of atmospheres dangerous to man (Haldane, 1895b).

In the human being the rate at which the body takes up carbon monoxide depends mainly on two factors, the partial pressure of carbon monoxide in the air breathed, and the volume of pulmonary ventilation; it also depends on the rate of blood flow through the lungs, but under normal conditions the blood flow and the pulmonary ventilation usually vary together. It is important to know how fast the percent-

age saturation of the blood will rise when an individual is exposed to a given concentration of carbon monoxide in air, in order to predict the nature of the symptoms and to decide the limits of safety. When a subject at rest inhales a mixture of air and carbon monoxide the saturation of his blood with carbon monoxide increases in a regular manner. Haldane (1935a) has described this process for the saturation of the tissues with nitrogen when man is subjected to increased atmospheric pressure, and the saturation of hemoglobin with carbon monoxide proceeds in a similar way in the living body (Killick, 1936). Figure 1[1] shows the form of the curve relating the percentage saturation of the blood to the duration of exposure; this curve may be applied to any

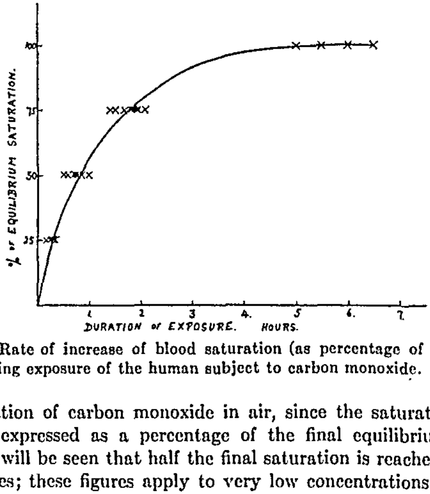

Fig. 1. Rate of increase of blood saturation (as percentage of equilibrium value) during exposure of the human subject to carbon monoxide.

concentration of carbon monoxide in air, since the saturation of the blood is expressed as a percentage of the final equilibrium saturation. It will be seen that half the final saturation is reached in about 47 minutes; these figures apply to very low concentrations of carbon monoxide, and Henderson, Haggard et al. (1921a) found that whereas half the final saturation was reached within one hour when the atmospheric concentration of carbon monoxide did not exceed 0.07 per cent, above this concentration the percentage saturation of the blood seemed to rise somewhat more rapidly. If one knows approximately

[1] The data embodied in this diagram were obtained from the experiments reported in 1936, but have not previously been recorded in this form.

what the equilibrium constant for the blood is, it is easy to calculate what will be the final percentage saturation with carbon monoxide for any given partial pressure of the gas, and so one can deduce the rate at which carbon monoxide will be absorbed. It is also possible to predict approximately how long any given concentration of carbon monoxide can be breathed before symptoms of poisoning appear, a point of obvious practical importance. It must be remembered that when an individual is doing muscular work the process of absorption is more rapid; according to Henderson and his co-workers (Henderson, Haggard, Teague, Prince and Wunderlich, 1921a) absorption is twice as rapid during moderate exercise (walking) as it is while sitting at rest, and three times as rapid during fairly hard work.

The second method depends on the observation that when an individual begins to breathe a mixture of carbon monoxide and air, about half the carbon monoxide taken into the lungs with each breath is retained (Haldane, 1895b; Veale, 1922). This, of course, only holds during the early period of breathing such a mixture, while absorption is relatively rapid, and the proportion of carbon monoxide retained increases somewhat when breathing is deeper during exercise. The knowledge of the volume of carbon monoxide absorbed, thus obtained, together with the total gas capacity of the individual's circulating blood, enables one to calculate the rate at which the percentage saturation of the blood will rise during inhalation of any given mixture of carbon monoxide and air. Assuming a blood volume of 5 liters, and a pulmonary ventilation of 7 liters per minute, Haldane (1895b) states that the time required for the production of symptoms in an adult man depends on the time required for the inhalation of about 660 cc. carbon monoxide, or the absorption of about 330 cc.

It is important to remember that the reaction

$$HbO_2 + CO \rightleftharpoons HbCO + O_2$$

is a reversible one, and therefore the carbon monoxide gradually disappears from the circulating blood when a poisoned man or animal breathes fresh air. The rate of elimination of carbon monoxide is governed by the same factors as is its absorption. When an individual whose blood is, for example, 30 per cent saturated with carbon monoxide, is brought into fresh air, carbon monoxide is eliminated and the percentage saturation falls fairly rapidly at first (to 20 per cent in less than two hours (Sayers, Yant, Levy and Fulton, 1929)), but the process slows down as the percentage saturation decreases, and it is

usually many hours before the last traces of carbon monoxide disappear. Sayers and his co-workers found about 5 per cent saturation 7 to 8 hours after the end of the exposure. The elimination of carbon monoxide is hastened by any factor that increases the volume of pulmonary ventilation and the circulation rate of the blood, or that raises the partial pressure of oxygen in the alveoli (Stadie and Martin, 1925-26).

It has been claimed that ultra-violet light hastens the elimination of carbon monoxide from the blood; Koza (1929) poisoned rabbits with illuminating gas to the stage of unconsciousness, and found that in the irradiated animals the percentage of carboxyhemoglobin was reduced by $\frac{3}{4}$ in a period during which the controls lost only $\frac{1}{4}$ of their carboxyhemoglobin. He suggests that the effect is due to dissociation of carboxyhemoglobin in the skin capillaries under the influence of the radiation. This explanation is rather difficult to accept unless one assumes that the carbon monoxide is eliminated through the skin, and it seems possible that the difference between the radiated animals and the controls was due to greater activity of the former, a factor that is very liable to falsify such experiments (Haggard and Greenberg, 1933).

Mode of action of carbon monoxide. The percentage saturation of the blood with carbon monoxide represents fairly accurately the degree of anoxemia to which the individual is subjected, because carbon monoxide acts by preventing the normal carriage of oxygen in the blood, and not as a direct tissue poison. It is known that carbon monoxide can act as a tissue poison by inhibiting the enzymic oxidative systems in the tissues, but this effect is only evident at relatively high partial pressures of the gas. Warburg (1926) showed that the respiration of yeast is inhibited in a mixture of carbon monoxide and oxygen, the degree of inhibition depending on the ratio of carbon monoxide to oxygen, but inhibition was not evident until the partial pressure of the carbon monoxide was nearly 5 times as high as that of the oxygen; De Meio, Kissin and Barron (1934) compared the metabolism of isolated tissues in mixtures of oxygen with carbon monoxide and with nitrogen, and found that certain tissues did show inhibition with carbon monoxide. This effect was demonstrated in the living body by J. B. S. Haldane (1927); he repeated J. S. Haldane's experiment (1895a) by exposing rats to a total pressure of two atmospheres of oxygen and one atmosphere of carbon monoxide. The blood of the animals was 98.3 per cent saturated with carbon monoxide under these conditions, but they remained almost unaffected because the blood carried an amount

of oxygen in simple solution that was sufficient for the needs of the body at rest. When, however, another atmosphere of carbon monoxide was added severe symptoms were evinced, and Haldane concluded that the carbon monoxide, at this high partial pressure, affected some substance in the tissues. It is clear from the first part of this experiment that the inhibitory effect on the tissues is of no significance under ordinary conditions in the living animal, because the deprivation of oxygen by the combination of carbon monoxide with hemoglobin would be fatal long before the partial pressure of carbon monoxide was high enough to affect tissue oxidative systems. Carbon monoxide acts, then, as a physiologically inert gas, except for its power of combining with hemoglobin, and this statement is confirmed by the indifference to carbon monoxide of animals and tissues without hemoglobin, e.g., cockroaches (J. S. Haldane, 1895a) cultures of chick neuroblastic tissue (Haggard, 1922). Carbon monoxide does not appear to be oxidized in the living body (J. S. Haldane and Smith, 1896; J. S. Haldane, 1900; M. Krogh, 1915), although Fenn and Cobb (1932a, b) have shown that it is oxidized by certain isolated tissues when they are placed in an atmosphere of 80 per cent carbon monoxide and 20 per cent oxygen. In addition to combining with the hemoglobin of the blood carbon monoxide combines with the muscle hemoglobin, but the affinity of the muscle hemoglobin for carbon monoxide is much lower than that of the blood hemoglobin. Theorell (1934) has shown that myoglobin is similar to hemoglobin in its combination with oxygen and with carbon monoxide, but the equilibrium constant that determines its partition between the two gases is only 13.8 for horse myoglobin at 37°C. (20.9 at 15°C.); Theorell notes that this value for K fits exactly on Barcroft's curve relating log K to "span," (see p. 315). When carbon monoxide is inhaled only a small proportion of the absorbed gas combines with the myoglobin; Gscheidlen (1869) stated that in estimating blood volume by the carbon monoxide method muscle hemoglobin took up about $\frac{1}{20}$ of the carbon monoxide absorbed. This is consistent with Whipple's observation (1926a) that in dogs the myoglobin is about 15 per cent of the total hemoglobin, taking into consideration the relatively low affinity of the myoglobin for carbon monoxide. There is no direct evidence as to the effects produced in the living body by a partial saturation of the myoglobin with carbon monoxide, but Millikan (1936) has demonstrated that myoglobin fulfills an important function as a short period oxygen store during muscular contraction, and Whipple (1926b) found that the quantity of hemoglobin in the skeletal muscles

was dependent on the degree of muscular activity. It is interesting that muscular weakness is a prominent symptom of acute carbon monoxide poisoning (see p. 339), although there is no evidence of any connection between this symptom and the combination of carbon monoxide with the muscle hemoglobin.

Barkan (1938) has described a pigment which he calls pseudohemoglobin, and which is an intermediate stage between hemoglobin and bile pigment. It accompanies hemoglobin to the extent of 5 per cent, and its relative affinity for carbon monoxide is significantly higher than that of hemoglobin. Barkan claims that the properties of this pigment are useful in diagnosing carbon monoxide poisoning, and may prove useful in elucidating some of the problems of chronic poisoning. The presence of this pigment may account for the recorded persistence of carbon monoxide in the blood for periods of days after acute poisoning.

The symptoms of carbon monoxide anoxemia in man. The symptoms of acute carbon monoxide poisoning in man have often been described (Glaister and Logan, 1914a; Sayers and Davenport, 1930; Haldane and Priestley, 1935b; Drinker 1938a), and may be considered in relation to the degree of saturation of the blood with carbon monoxide. Table 1 indicates the sequence of symptoms.

As indicated in table 1 the symptoms are accentuated by muscular exercise, and serious symptoms appear at a lower percentage saturation of the blood when the individual is working than when he is quiet. The symptoms vary also with the rate at which the percentage saturation of the blood is increasing. A man who is exposed to a high concentration of carbon monoxide, as for example after a colliery explosion, may experience very few symptoms until muscular weakness supervenes, and he is near unconsciousness. During exposure to low concentrations of carbon monoxide the symptoms appear gradually and their severity varies with the duration of exposure. If the percentage saturation of the blood with carbon monoxide is raised fairly rapidly, say to 35 per cent, and then maintained at that level for a short period (10–20 min.) the symptoms and after effects will be slight. If, instead, the carbon monoxide is gradually absorbed, so that the 35 per cent saturation represents equilibrium and the whole process occupies 6 or 7 hours, the result will be a severe headache. After an exposure of this type the symptoms are usually most severe during the period of recovery, and the headache that is characteristic of carbon monoxide anoxemia often does not begin until after the period of exposure to the gas.

It is interesting that when an individual is taken up to a high altitude by railway, there is usually a latent period of several hours before the symptoms of mountain sickness appear (Haldane and Priestley, 1935c). The relation of the symptoms and after effects of inhalation of carbon monoxide to the duration of exposure as well as to the atmospheric

TABLE 1

PER CENT SATURATION OF BLOOD WITH CO.	SYMPTOMS
0–10	Nil
10–20	Nil at rest; during exercise tightness across forehead, slight headache
20–30	Frontal headache may occur at rest; exertion causes dizziness, palpitation, and hyperpnea
30–40	At rest, headache, frontal or occipital, increased pulse rate, deeper breathing, palpitations, nausea; exertion causes dizziness, dimness of vision, abnormal increase of pulse rate and respiration, sometimes collapse
40–50	All symptoms more marked; vision, hearing, and intelligence begin to be impaired; muscular weakness on attempted exertion, with greater likelihood of collapse. Nausea and vomiting
50–60	Syncope; increased respiration and pulse rate; coma
60–70	Coma with depressed heart action and respiration; possibly death
70–80	Weak pulse and respiration; respiratory failure and death

The data in this table are taken from the sources mentioned above, and from Sayers, Yant, Levy and Fulton (1929) and Killick (1936).

concentration has been quantitatively expressed by Henderson, Haggard, et al. (1921b) as follows:

When duration of exposure in hours $\times$ concentration in parts per 10,000
= 3; effect is nil
= 6; effect just perceptible
= 9; headache and nausea
= 15; dangerous

This quantitative statement provides a very useful guide in assessing quickly the probable effects of any given exposure, but of course it does not allow for the fact that very low concentrations of carbon monoxide would never produce symptoms because the percentage saturation of the blood at equilibrium would be too low.

The symptoms of carbon monoxide poisoning resemble closely the symptoms of anoxemia due to altitude, or to a low partial pressure of oxygen produced in any other way, but there is one marked difference. In carbon monoxide poisoning syncope, or a feeling of faintness, is much more prominent than hyperpnea, whereas the opposite may be said of the anoxemia due to imperfect oxygenation of the arterial blood associated with a low partial pressure of oxygen. Haldane and Priestley (1935b) consider that this difference indicates that the respiratory center responds to the oxygen tension in the arterial blood, which is at its normal level in carbon monoxide anoxemia. The heart is affected, on the other hand, by the reduced quantity of oxygen obtained from the blood in carbon monoxide anoxemia, with the result that exertion causes a fall of blood pressure and faintness. The headache which results from carbon monoxide anoxemia appears to be due to an increased pressure in the cerebro-spinal fluid; there is evidence that such an increase accompanies the headache, and that measures designed to reduce the pressure also relieve the headache (Forbes, Cobb and Fremont-Smith, 1924). These workers record that in one case after the exposure to carbon monoxide, when the headache was worst, ophthalmoscopic examination showed that congestion of the retinal vessels was at a maximum. Forbes et al. (1924) also observed an increased cerebro-spinal fluid pressure, congestion of cerebral vessels, and cerebral edema in dogs and cats during inhalation of carbon monoxide.

Nausea and vomiting are symptoms common to more than one type of anoxemia; they occur in mountain sickness, as the name suggests, and they also occur in carbon monoxide anoxemia. Some degree of nausea is often coexistent with the headache, and vomiting may be precipitated by slight exertion (Killick, 1936).[2] Nausea and vomiting, like the headache, may be at their worst during the recovery period. All these points suggest that the vomiting is due to direct stimulation of the nerve center which controls it, either by the local effects of oxygen lack, or by the edema following the anoxemia.

The symptoms of carbon monoxide anoxemia are strikingly different from those of anemia with a corresponding reduction in hemoglobin; a man whose blood is 50 per cent saturated with carbon monoxide is almost helpless, but a man whose blood contains only 50 per cent of the normal quantity of hemoglobin behaves normally, and can often carry on his work. The partial pressure of oxygen in the arterial blood is

[2] Unpublished observations.

much the same in the two cases, and the quantity of oxygen carried is the same, but the dissociation of oxygen from the oxyhemoglobin is quite different. Douglas, Haldane and Haldane (1912) showed that when the blood is partially saturated with carbon monoxide the dissociation curve of the remaining oxyhemoglobin is altered in such a *way that the oxygen dissociates at a much lower partial pressure than* usual. This means that the oxygen tension in the tissues must fall much lower than it would if the oxygen dissociation curve were unaltered. J. B. S. Haldane (1912–1913) has published dissociation curves for the oxyhemoglobin remaining in blood that is saturated to various degrees with carbon monoxide which demonstrate this point. Stadie and Martin (1925–1926) made it still clearer by comparing the oxygen dissociation curve of blood containing 40 per cent of the normal quantity of hemoglobin with that of the oxyhemoglobin in blood that was 60 per cent saturated with carbon monoxide. They point out that the alteration in shape and position of the dissociation curve causes a marked lowering of the partial pressure at which oxygen is available for tissue metabolism, and therefore profound anoxemia occurs in carbon monoxide poisoning although the blood may contain 2 or 3 times the amount of oxygen necessary for normal tissue function.

Effects of carbon monoxide anoxemia. There are innumerable clinical records of carbon monoxide poisoning, and a multiplicity of aftereffects has been ascribed to it, most of which represent the effect of anoxemia on various parts of the body. The literature on this subject has been summarized by Glaister and Logan (1914b), Sayers and Davenport (1930), and Drinker (1938b).

Central nervous system. The central nervous system is, of course, the tissue most easily damaged by anoxemia, and it shows many changes as a result of carbon monoxide poisoning. After death from carbon monoxide poisoning the brain is found to be hyperemic and edematous, usually showing numerous punctate hemorrhages (Mott, 1907; Hill and Semerak, 1918). Mott (1907) and Hiller (1924) suggest, on the basis of postmortem examination of brains from cases of carbon monoxide poisoning, that these appearances are due to a toxic action of the carbon monoxide on the capillaries. Degeneration of nerve cells is commonly found in the cerebral cortex and in the globus pallidus of the lenticular nucleus (Hill and Semerak, 1918; Hadfield, 1929; Christiani, 1934). Bilateral softening of the globus pallidus is so frequently found as to be characteristic of death from carbon monoxide poisoning; Hill and Semerak found obvious softening in 14 out of 32 cases, and micro-

scopical evidence of degeneration in every case. Meyer (1928) records a symmetrical affection of the pallidum as an extremely regular finding in dogs poisoned with carbon monoxide. Kolisko (1914) put forward an explanation of this localization on the basis of the anatomical arrangement of the arteries to the part, making the pallidum peculiarly liable to anoxemia. Hadfield (1929), in a small series of cases, found that this focal degeneration was associated with vascular siderosis of the pallidum, and concluded that "vascular siderosis predisposes to the acute bilateral destruction of the globus pallidus which is frequent in coal-gas poisoning." Schäffer (1903) and Hsü and Cheng (1938) have drawn attention to necrosis of nerve fibers both in the central nervous system and in peripheral nerves; Hsü and Cheng describe a diffuse reaction in the deeper cerebral white matter which is not uncommon in carbon monoxide poisoning.

An extensive pathological investigation was carried out on dogs by Yant, Chornyak, Shrenk, Patty and Sayers (1934); in dogs exposed to a concentration of carbon monoxide that killed in 20 to 30 minutes the brain showed considerable edema, with dilated blood vessels and some petechial hemorrhages, especially in the cerebral cortex and corpus striatum. The nerve cells were severely damaged, the most affected being those of the cortex, corpus striatum, dorsal motor nuclei of the vagi, and the dorsal sensory areas of the medulla. When the concentration of carbon monoxide was lower, so that the dogs died after 7 to 15 hours, the pathological changes differed only in degree from those observed in animals that survived only 20 to 30 minutes. The edema and the circulatory changes were more marked, and the degenerative changes in the nerve cells had progressed farther, but the areas showing the most severe effects were still the same. The third group of dogs was exposed to a concentration of carbon monoxide that produced a blood saturation of 65 to 75 per cent; this condition was maintained for 15 hours, at the end of which period the dogs appeared moribund. They were then removed from the poisonous atmosphere and allowed to recover; some of the dogs died within a few days and the others were killed at various intervals. There was a great variation in the pathological changes observed in the different animals; the most marked lesions occurred in 2 dogs killed 62 and 65 days after exposure, while the changes exhibited by animals killed before and after these two were less severe. In general the findings in these animals were extensive proliferation of neuroglia and endothelium, large cystic areas in the medullary substance of the brain, with chromatolysis or frag-

mentation of some of the nerve cells; there were also focal areas of myelin degeneration throughout the nervous system, including the peripheral nerves. These results were compared with the effects of simple O_2 lack, and the pathological findings were very similar. This similarity seems to conflict with the view that carbon monoxide has a toxic effect on the capillaries (see p. 324), and suggests that the damage to blood vessels results from oxygen lack.

These experimental findings are in agreement with the pathological changes observed in human beings killed by carbon monoxide. But in spite of these serious changes in the central nervous system permanent mental or neurological disease is a rare sequela of carbon monoxide poisoning; Shillito, Drinker and Shaughnessy (1936) examined the records of 21,143 cases of carbon monoxide poisoning occurring during 10 years in New York, and found only 39 patients suffering from mental or neurological after-effects. All of these 39 patients had been severely poisoned, all were unconscious when found, and 14 of them were described as "in extremis."

Susceptibility to infection. It has often been claimed that carbon monoxide poisoning increases susceptibility to infection. The evidence adduced in support of this statement is that pneumonia frequently follows severe acute carbon monoxide poisoning; this subject is discussed very fully by Glaister and Logan (1914c), who quote an impressive series of recorded deaths from pneumonia after severe carbon monoxide poisoning. Löwy (1925) suggested that as a result of the tissue anoxemia either resistance to infection is lowered, or the virulence of bacteria is raised. Drinker (1938c) is skeptical of the suggestion that carbon monoxide has any specific effect in increasing susceptibility, and considers that the incidence of pneumonia is associated with a long period of unconsciousness, whatever the cause.

It has also been stated that long continued exposure to low concentrations of carbon monoxide predisposes to infection, and Sudhues (1932) states that it is generally accepted that chronic carbon monoxide poisoning causes diminished resistance to infection, but in experimental work on rabbits she could find no difference in the blood content of natural complement, agglutinins, or in phagocytosis, between her controls and animals exposed daily to carbon monoxide over a period of 2 years, nor did she find any difference in the production of specific antibodies to typhoid bacilli. Buresch (1933), on the other hand, did find a loss of hemolytic complement in rabbits exposed daily to carbon monoxide, and also a lowered bactericidal index in dogs similarly

treated. There seems to be little evidence that susceptibility to infection is increased in human beings as a result of repeated exposure to low concentrations of carbon monoxide; Beck (1936) does not mention this as one of the symptoms observed in his series of 97 patients who had been subjected repeatedly to sub-lethal doses of carbon monoxide, and Sayers and his co-workers (1929) record no indication that repeated exposures under experimental conditions were deleterious to the health of their subjects. Löwy (1926), however, cites diminished resistance to infection as one of the effects of chronic exposure to carbon monoxide.

Cardio-vascular system. The heart shows comparatively little aftereffect from carbon monoxide anoxemia; during the period of anoxemia the heart rate at rest is unaffected until the saturation of the blood reaches 25 to 30 per cent (Sayers, Yant, Levy and Fulton, 1929; Killick, 1936; Forbes, Dill, de Silva and Van Deventer, 1937). After this the pulse rate increases approximately in proportion to the saturation of the blood with carbon monoxide (Killick, 1936), but as the anoxemia becomes more severe the heart begins to fail. Exercise, when the blood is partially saturated with carbon monoxide, causes an abnormal increase in pulse rate.

Drinker (1938d) considers that individuals with antecedent heart disease are killed immediately as a result of acute poisoning by carbon monoxide, or they survive only a short time, whereas individuals with thoroughly sound hearts may develop chronic heart disease as a sequela of acute poisoning, although this happens only very rarely. It is not infrequent to find the signs of a dilated heart without any organic damage after acute poisoning with carbon monoxide (Zondek, 1919, 1920; Haldane and Priestley, 1935e), and in these cases the heart muscle often recovers completely. Stearns, Drinker and Shaughnessy (1938) took electrocardiograms in 22 cases of acute poisoning, the patients being examined after the period of anoxemia was over, and found changes in the T wave and in the S-T segment in most of the patients, but disturbances of rhythm and conduction were rare. Disturbances of conduction have been produced in animals by severe asphyxia (Lewis, White and Meakins, 1913–1914; Haggard, 1921) but Haggard states that the oxygen deficiency caused by carbon monoxide is not in itself sufficient to cause impairment of auriculo-ventricular conduction, which is only induced by the added anoxemia of respiratory failure.

In recurrent exposure to carbon monoxide, palpitation is a frequent complaint, sometimes accompanied by cardiac pain (Von Dassel, 1932; Beck, 1936), and irregularities of rhythm are also described (Löwy,

1926). Beck (1936) found a slowing of the pulse rate and lowering of blood pressure in 50 per cent of his cases of chronic exposure, but Ciampolini (1924) records a tendency to increased pulse rate under similar conditions. It is not quite clear, however, whether Ciampolini refers to the resting pulse rate, so the discrepancy may be only apparent. In a series of 136 cases of chronic carbon monoxide anoxemia Beck and Suter (1938) found that myocardial symptoms, e.g., anginal attacks, were frequently manifested. As in the production of lesions in the brain, the affection of the heart was primarily vascular, and coronary thrombosis was not infrequent. Argyll Campbell (1929) considers that the ability to tolerate prolonged exposure depends on the response of the heart; he found atrophy of parts of the heart muscle in some of his animals after prolonged exposure to carbon monoxide, and he also describes a hypertrophy of the heart muscle in mice (1932).

Glaister and Logan (1914d) state that during inhalation of carbon monoxide the blood pressure first rises, with a feeling of fulness and throbbing in the head, but later falls to a low level. Brewer (1937) found that in dogs pure carbon monoxide did not produce the rise in blood pressure that breathing nitrogen did, and that carbon monoxide in air caused a fall of blood pressure, but the experimental conditions were so extreme that it is doubtful whether these findings have any significance in relation to the effect of breathing the usual relatively low atmospheric concentrations of carbon monoxide.

A number of cases are on record where a man has been exposed to carbon monoxide, but without very severe immediate symptoms, and yet has collapsed and died some minutes or hours later (Glaister and Logan, 1914e; Drinker, 1938e); such sudden death is usually cardiac in origin, and is of some medico-legal interest, for death may occur under circumstances suggesting foul play.

The reference to thrombosis in the coronary vessels, and also in the cerebral vessels, is interesting in view of the old controversy as to whether the blood is more fluid than usual after death from carbon monoxide. It is often claimed that the blood is abnormally fluid at death, and does not readily coagulate (Glaister and Logan, 1914f; Ramsey and Eilmann, 1931), while on the other hand Rambousek (1913) states that carbon monoxide favors coagulation. Forbes and Hompe (1921) attempted to obtain evidence on this point by measuring the clotting time of the blood in cats poisoned fairly rapidly with carbon monoxide; they found no alteration in clotting time and no change in prothrombin content. It seems likely that the hemorrhages

and thrombi found after fatal carbon monoxide anoxemia are due to changes in the capillary walls rather than in the blood itself (Drinker, 1938f). Semerak and Bacon (1930) have summarized the literature dealing with changes in the vessel walls, and conclude that various degenerative changes may follow severe carbon monoxide anoxemia.

Metabolism. Carbon monoxide anoxemia, as would be expected, affects the metabolism in several ways. Walters (1927) showed that in white rats continuously exposed to low concentrations of carbon monoxide the metabolic rate was diminished even in the early stages of anoxemia (16 to 35 per cent saturation of the blood), and that this fall in metabolic rate could be correlated with the symptoms of poisoning. Haldane (1895a) demonstrated the same effect in mice. On the other hand Schulze (1936a), using white mice, and Reploh (1932), using rabbits, observed that repeated exposure to carbon monoxide caused an increase in metabolic rate. Schulze (1936a) describes changes in the thyroid in his animals of a type usually associated with increased activity. Kampelmann and Schulze (1937) extended this investigation by observing that in guinea pigs repeatedly exposed to carbon monoxide there was a diminution of the thyrotropic hormone of the pituitary, which appeared to be secondary to the activation of the thyroid. Yet a third type of result is that recorded by Haggard and Henderson (1921); they found that when dogs were rapidly gassed the oxygen consumption did not fall, but either remained unaltered or increased. They attributed the increase, when it occurred, to the increased activity of the respiratory muscles involved in the considerable degree of hyperpnea that was associated with the anoxemia.

Summing up these experimental findings, it seems that during exposure to low concentrations of carbon monoxide, when the anoxemia develops slowly, and is not so severe as to cause collapse and death, the metabolic rate is slowed; when the anoxemia is rapid and severe, as in Haggard and Henderson's experiments, this effect does not develop and the metabolic rate may actually rise as a result of increased activity of the respiratory muscles. Repeated severe poisonings, as in Reploh and Schulze's experiments, appear to affect the ductless glands in such a way that the metabolism is increased; this increase, it must be emphasized, was observed between or following the successive periods of anoxemia and not during these periods, as was the case in Haldane's and Walters' experiments. The only relevant observation on human subjects seems to be that of Beck (1936) who found that the metabolic rate was significantly reduced in just over half of his patients who had

been repeatedly exposed to carbon monoxide, and secondly, the observation frequently made that in acute poisoning there is a very marked fall of body temperature (Drinker, 1938g), presumably due to depressed metabolism.

Several workers have recorded an increased urinary excretion of total nitrogen and of NH_3 in carbon monoxide poisoning (Glaubitz, 1921; Tscherkess and Melnikova, 1928). Tscherkess and Melnikova subjected various animals to short periods of severe carbon monoxide anoxemia, and found that both urinary nitrogen and inorganic phosphorus were increased during and after the poisoning, returning to normal after 1 to 3 days. It is claimed that this indicates increased destruction of protein, and a raised metabolic rate, but apparently no other method of estimating the metabolic rate was employed.

Glycosuria often occurs as a result of carbon monoxide anoxemia, as was first described by Claude Bernard (1857), and seems to be due to the mobilization of the liver glycogen, as it does not occur when starved animals that have lost their liver glycogen are subjected to anoxemia (Araki, 1891). Kellaway (1919) considers that the mobilization of sugar is an effect of anoxemia on the central nervous system, although accelerated output of adrenin may play a part. Schulze (1936b), in his experiments on mice, found that the blood sugar rose steeply as a result of each exposure to carbon monoxide, and returned gradually to normal in about 3 hours; the liver glycogen diminished with successive exposures, indicating the source of the blood sugar. Buresch (1933) also observed an increased blood sugar in rabbits exposed daily to carbon monoxide, although Boedicker (1933) could find no definite evidence that these repeated exposures altered the form of the blood sugar curve following a dose of glucose by stomach tube. Mikami (1926–27) records that in rabbits the rise of blood sugar resulting from administration of carbon monoxide was proportional to the degree of anoxemia; he also found that intravenous injection of alkali inhibited the hyperglycemia, and also prevented the fall in arterial carbon dioxide content associated with acute poisoning (see p. 331). This connection suggests that changes in the acid-base balance of the blood may play a part in the production of hyperglycemia.

Respiration and chemical changes in the blood. A number of conflicting views have been expressed as to the effect of carbon monoxide anoxemia on the acid-base equilibrium of the blood, but most observers have found a decreased alkali reserve (Haggard and Henderson, 1921; Mikami, 1926–27; Tscherkess and Melnikova, 1928; Kamei, 1931),

although their interpretations of this result vary. Haggard and Henderson exposed dogs to 0.25 per cent carbon monoxide; during the period of anoxemia the respiration increased greatly, the volume sometimes being three times as great as before the experiment started, the carbon dioxide combining power of the arterial blood fell gradually while its carbon dioxide content fell more rapidly. The hyperpnea was prevented by section of both vagi. From these results they concluded that the hyperpnea caused by anoxemia produced an alkalosis by washing out carbon dioxide, and that the alkali reserve fell as a result of the alkalosis. The absence of hyperpnea after vagal section they regard as evidence that "oxygen deficiency by itself does not directly cause in the tissues and blood an increased production of organic acids." Neither Mikami nor Kamei, however, was able to confirm this effect of vagal section. Both these workers used hypodermic injection as a method of administering carbon monoxide; Mikami observed a very marked fall in arterial carbon dioxide content in rabbits, but since he only measured the respiratory rate, and not the volume, his statement that the increased breathing was not sufficient to account for the fall in carbon dioxide is not very convincing: he found that the plasma pH was definitely lowered as a result of carbon monoxide anoxemia. Kamei also observed an increased ratio of dissolved to combined carbon dioxide in the blood of dogs similarly treated. Mikami and Kamei both administered carbon monoxide by inhalation as well as by injection, and claim that the results were very similar. Haggard and Henderson noticed in their dogs that during the early recovery period the respiration was depressed, with resultant retention of carbon dioxide, and development of acidosis. It seems possible that the diminished pH observed by Mikami and Kamei was actually recorded during such a period of depressed respiration, especially as it could not be easy to determine the stage of maximum anoxemia after injection of carbon monoxide.

In human beings hyperpnea does not seem to be such a prominent symptom of carbon monoxide poisoning; Haldane (1895b) in experiments on himself noted that hyperpnea began to appear when his blood was about 30 per cent saturated with carbon monoxide, and became more marked as the saturation increased. Sayers and his co-workers (1929) state that in their series of experiments on human subjects there was no change in the respiration as a result of exposure to 0.02 to 0.04 per cent carbon monoxide. It is interesting that Kamei (1931) states that in his dogs inhalation of 1 per cent carbon monoxide

caused hyperpnea, whereas inhalation of 0.05 to 0.08 per cent did not; in the experiments referred to above when Haldane experienced hyperpnea he was breathing fairly high concentrations of carbon monoxide (0.12 per cent–0.50 per cent), while Sayers' subjects only breathed low concentrations. Unfortunately in these human experiments no estimations were made of carbon dioxide combining power or carbon dioxide content of the blood, but the available evidence supports the hypothesis that the diminished alkali reserve is associated with hyperpnea, and is strictly comparable with the change caused by the anoxemia of high altitude.

Reproduction and sex glands. Carbon monoxide anoxemia seems to affect the gonads; McCombs (1912) records that men surviving acute poisoning are sometimes impotent, and Drinker (1938h) has seen such a case; Rossiter (1928) refers to loss of sexual desire after continual exposure to carbon monoxide in sub-lethal doses, and Williams (1930) states that poisoning may cause death and expulsion of the fetus. Nicloux (1901), in his work on the passage of gases across the placenta, records that during slow poisoning of pregnant guinea pigs with carbon monoxide the fetal and maternal bloods contained the same quantity of carbon monoxide, but that when exposed to a high concentration of carbon monoxide the guinea pigs died before an appreciable amount of carbon monoxide had reached the fetal blood, although the fetus suffered from deprivation of oxygen.

There is considerable experimental evidence that prolonged exposure to low concentrations of carbon monoxide greatly reduces fertility in animals (Campbell, 1934 (mice); Williams and Smith, 1935 (rats); Patterson, Smith, and Pickett, 1938 (mice)). Buresch (1933), working with rabbits and mice, found that repeated exposures to carbon monoxide caused a tendency for the females to abort. Williams and Smith used a concentration of carbon monoxide sufficient to produce 60 to 70 per cent saturation of the blood in the course of an hour; daily exposures affected the females so that they first produced inferior offspring, later dead feti, and finally pregnancies ceased. The males after 75 daily exposures were apparently sterile, the sperm cells being non-motile or absent; the testes were reduced to $\frac{1}{3}$ to $\frac{1}{2}$ the weight of the controls. These results were confirmed by Patterson, Smith and Pickett, who also observed that in the males the hypophysis became highly basophilic and contained many vacuolated cells (such as are seen after castration); in addition they demonstrated an increase in the content of gonadotropic hormone. It should be noted that Williams and Smith, and

Patterson and his co-workers used illuminating gas as a source of carbon monoxide; this gas has been shown to be somewhat more toxic than its content of carbon monoxide indicates (Haggard, 1922; Henderson, Haggard, Teague, Prince and Wunderlich, 1921b), and it is possible that this may account in part for their results.

Acclimatization. Certain adverse effects of continuous or repeated exposure to carbon monoxide have already been described, but another result of such treatment must now be discussed; the response of the healthy man or animal is to develop some degree of acquired tolerance of the gas, although adverse effects may show themselves at the same time. This acquired tolerance or acclimatization to carbon monoxide has been recognized for a considerable period among those whose work exposes them to carbon monoxide (Faure, 1856; Glaister and Logan, 1914g; Oliver, 1916; Official History of the War, 1923), and its occurrence has been confirmed more recently by experimental methods.

The first systematic attempt to find an explanation of the phenomenon was made by Nasmith and Graham (1906). They kept guinea pigs continuously in an atmosphere that produced a 25 per cent saturation of their blood with carbon monoxide; after the first few days the animals appeared quite normal, but the red cell count and the hemoglobin content of their blood gradually rose in the course of 3 to 4 weeks from an average value of 5.88 million red cells and 88 per cent hemoglobin, to 7.96 million red cells and 105 per cent hemoglobin. The concentration of carbon monoxide in the air was then increased to give 35 per cent saturation of the blood, and the red cell counts rose again to an average of 9 million. Finally the saturation of the blood was put up to 45 per cent and the animals at first showed symptoms of poisoning, but they recovered in a few days and the red cell count eventually reached an average of 10.5 million with a hemoglobin index of 110 per cent. The degree of acclimatization was indicated by the observation that fresh guinea pigs placed in this atmosphere only survived a few days, whereas the acclimatized animals appeared to be unaffected. Nasmith and Graham record that degenerative changes in the red cells preceded the erythrocytosis, and that some degree of eosinophilia was observed. These experiments yielded no evidence as to whether the percentage saturation of the blood with carbon monoxide altered as acclimatization developed.

Argyll Campbell (1929–1930, 1933, 1934) carried the investigation of the problem a stage further, and showed that rabbits, rats, mice and guinea pigs could tolerate a much higher atmospheric concentration

of carbon monoxide after acclimatization by continuous exposure than they could when unacclimatized. He confirmed in these animals the observation of polycythemia made by Nasmith and Graham. He attempted to follow the progress of acclimatization by estimating the tensions of oxygen and carbon monoxide in the tissues during exposure of rabbits to carbon monoxide. The tension of carbon monoxide maintained a fairly constant relationship with the concentration of the gas in the air breathed as acclimatization developed, but in the acclimatized animals the tissue oxygen tension did not fall quite so low as it did in the unacclimatized when both had their blood saturated to the same extent with carbon monoxide; this was probably a result of the increased oxygen capacity of the blood. Campbell's data for the percentage saturation of the blood with carbon monoxide indicate that its relationship with the atmospheric concentration of carbon monoxide remained unaltered as acclimatization developed. Campbell (1934) considers that a strict criterion of acclimatization should be adopted, and requires evidence of the maintenance of growth, body weight, appetite, general well-being and fertility. Perhaps the term "tolerance" should be used to describe any alteration short of this complete acclimatization, but it is usual to describe the disappearance of positive symptoms of poisoning as acclimatization.

Killick (1937) confirmed these observations on the effects of continuous exposure of mice to carbon monoxide. She found a considerable polycythemia, with a marked increase in the proportion of reticulocytes; there was enlargement of the spleen and some evidence of an increased blood volume. The reaction of the spleen is interesting, since there is evidence that this organ plays a considerable part in acute poisoning. Barcroft and Barcroft (1923) and Hanak and Harkavy (1924) have recorded a discrepancy between the carbon monoxide content of the spleen blood and of the circulating blood during exposure of rats and guinea pigs. The spleen blood apparently takes up carbon monoxide more slowly than the blood in the general circulation, and the time taken for the spleen pulp to attain equilibrium with the blood varied up to 6 hours.

De Boer and Carroll (1924–25) showed that in cats the spleen contracted when the animals were exposed to carbon monoxide, the contraction beginning when the percentage saturation of the blood with carbon monoxide was quite low. This contraction would presumably have the effect of expelling into the circulation blood from the spleen pulp whose carbon monoxide content was lower than that of the cir-

culating blood. That this mechanism is a useful one was shown by Barcroft, Murray, Orahovats, Sands, and Weiss (1925), when they compared the reaction of normal and splenectomized guinea pigs to carbon monoxide. The splenectomized animals succumbed in $\frac{3}{4}$ of the time of the normal animals.

It is not easy to see how this train of events could be of use when the animals live in an atmosphere containing carbon monoxide, but the enlargement of the spleen observed by Killick may have been connected with the polycythemia and increased blood volume. Muira's suggestion (1936) that the spleen removes from the circulation the erythrocytes containing carboxyhemoglobin is difficult to reconcile with the observation of a lag in the percentage saturation of the spleen pulp behind that of the circulating blood. It also assumes, apparently, that a red cell takes up oxygen or carbon monoxide, but not both, an assumption which hardly seems justified.

In the course of their estimations of the arterial oxygen tension by the carbon monoxide method Douglas and Haldane (1912) found that when the blood of mice became more than about 30 per cent saturated with carbon monoxide the arterial oxygen tension began to rise above that of the alveolar air, and Haldane and Priestley (1935f) consider that this power of secreting oxygen into the blood explains some of the phenomena of acclimatization to anoxemia. Killick (1937), in her experiments on mice, attempted to test this hypothesis by comparing the percentage saturation of the blood in vivo with the percentage saturation reached when the blood of the same animal was equilibrated in vitro with mixtures of air and carbon monoxide. The equilibration of several samples of blood from the same mouse provided the data for the calculation of the equilibrium constant, and when this value was known the equation

$$\frac{[HbCO]}{[HbO_2]} = \frac{K[CO]}{[O_2]}$$

could be used to calculate the arterial oxyten tension during life. The arterial oxygen tensions in the acclimatized mice, calculated in this way, fell within limits corresponding with the alveolar oxygen tension one might expect to find in mice. The conclusion was that under the conditions of the experiment acclimatization to carbon monoxide was not accompanied by any change in the absorption of carbon monoxide in the lungs, due to the secretion of oxygen.

Summing up the results of animal experiments, therefore, one can

state that animals develop a considerable degree of acclimatization to carbon monoxide, as a result of continuous or repeated exposure to low concentrations of the gas. This acclimatization is accompanied by an increased production of erythrocytes, thus raising the count in the circulating blood, and in certain modifications in the circulation, indicated by hypertrophy of the heart (Campbell, 1932), increased blood volume, and splenic changes. There is no evidence of any change in the relative affinity of hemoglobin for carbon monoxide (Killick, 1937), and the evidence as to some active process in the alveolar epithelium, such as secretion of oxygen, is conflicting.

Attempts to acclimatize human subjects by experimental methods have yielded somewhat conflicting results. Haldane (Haldane and Priestley, 1935g) records that he and Lorrain Smith became acclimatized during the course of a series of experiments in which they breathed carbon monoxide as a method of determining the arterial oxygen tension (Haldane and Smith, 1896). After a number of experiments they found it was necessary to breathe 0.06 per cent of carbon monoxide in order to produce the desired blood saturation of 30 per cent. Theoretically this concentration should produce about 55 per cent saturation of the blood; after a considerable lapse of time 0.04 per cent carbon monoxide was inhaled, and very severe symptoms were produced. Evidently the subject was acclimatized when breathing 0.06 per cent carbon monoxide, but had lost this acclimatization in the later experiment. The interesting point about this observation is that as a result of acclimatization there was a marked alteration in the relationship between the concentration of carbon monoxide inhaled and the percentage saturation of the blood.

Sayers, Yant, Levy and Fulton (1929) investigated the effect of repeated daily exposures to small amounts of automobile exhaust gas; the exhaust gas was used as a source of carbon monoxide and its concentration was adjusted to give the required concentration of carbon monoxide. The investigation was planned to give information as to the effects of exposure to exhaust gas such as traffic police and other officials would encounter when on duty in a road tunnel, and the observations made are so arranged as to make it difficult to conclude with certainty whether or not any acclimatization resulted. The six subjects were confined in an air-tight chamber of 1000 cu. ft. capacity for a period of 5 to 6 hours every day, and a continuous change of air was maintained during this time. Exhaust gas was added to the air in amounts sufficient to produce a concentration of 0.02 per cent, 0.03

per cent, or 0.04 per cent of carbon monoxide, the concentration being maintained constant throughout any one exposure. Control tests were interspersed amongst the carbon monoxide tests, in which no exhaust gas was added to the air.

The saturation of the blood with carbon monoxide was followed by taking blood samples at intervals from two of the subjects each day, and these results are recorded altogether, without any statement as to the subject whose blood was being examined, or the serial number of the exposure. After 8 control tests had been made 15 successive tests were carried out with 0.02 per cent carbon monoxide; during these tests the three subjects at rest experienced frontal headache towards the end of the exposure (in 22 per cent of the maximum number of occasions), while the other three, who were exercising moderately, experienced frontal headache slightly more frequently (28 per cent of the possible total). The recorded blood saturations are plotted against time of exposure on a single graph; the points are widely scattered, but since there is no indication of the source of the blood or the test during which it was taken one cannot judge whether the scatter represents the experimental error, the variation between the subjects, or the development of acclimatization. The lines which are drawn to represent the average blood saturation are of such a shape as to throw some doubt on the reliability of the method of estimating blood saturation (pyrotannic acid method). Two series of tests were made using 0.04 per cent carbon monoxide, and then a final series with 0.03 per cent carbon monoxide. In this final series the symptoms recorded were intermediate in severity between the exposures to 0.02 per cent and those to 0.04 per cent, which suggests that no acclimatization had occurred. The blood saturation attained in this final series corresponds fairly closely to the theoretical value. The effect of exercise was to hasten the absorption of carbon monoxide, and to increase the severity of the symptoms experienced.

During this series of exposures, extending over two months, the hemoglobin content of the blood of 5 of the 6 subjects increased, while the red cell count also increased in 4 subjects; the greatest increase was 20 per cent in the hemoglobin content and 1,000,000 in red cells.

There was no evidence of any deleterious effect on the health of any of the subjects.

A somewhat similar series of experiments, but performed on a single subject, was undertaken by Killick (1936) in an attempt to induce acclimatization under controlled conditions. The subject was exposed

to an atmosphere containing a low concentration of pure carbon monoxide in an air-tight chamber. The exposures were repeated at intervals of 4 to 7 days over a period of 3 to 4 months. The subject developed a considerable degree of acclimatization, as evidenced by the symptoms experienced. The blood saturation was followed during each exposure and it was found to increase in a regular manner, 25 per cent, 50 per cent and 75 per cent of the final equilibrium value being reached at approximately the same period of exposure, whatever the atmospheric concentration of carbon monoxide (see p. 317, fig. 1). The relationship between the final equilibrium value and the atmospheric concentration of carbon monoxide, however, altered as acclimatization developed. The extent of this alteration is indicated by the fact that the equilibrium value for the percentage saturation of the blood in the unacclimatized subject during exposure to 0.02 per cent carbon monoxide was the same as that observed during exposure of the acclimatized subject to 0.04 per cent carbon monoxide; furthermore the symptoms experienced on the two occasions were very similar. The value of the equilibrium constant for the subject's blood was determined in vitro, and was found to remain unaltered as acclimatization developed. These results, therefore, were due to some changed response of the living body, and two possible explanations were considered: 1, a selective activity of the alveolar membrane producing either a secretion of oxygen from the alveoli into the blood, or a hindrance to diffusion of carbon monoxide inwards; 2, removal of carbon monoxide from the blood by oxidative or other processes in the tissues.

The second explanation is improbable, since there is no evidence that the body can oxidize carbon monoxide (see p. 320). There are difficulties, also, in accepting the hypothesis of oxygen secretion; it has been claimed that the stimulus to oxygen secretion is an oxygen deficiency in the tissues (Haldane and Priestley, 1935h), but these experiments showed that the process underlying acclimatization was active when the blood was only 10 to 15 per cent saturated with carbon monoxide, a degree of saturation that is unlikely to be associated with oxygen lack in the tissues while the subject is at rest. This suggests that the explanation may be found in some process that is stimulated by the presence of carbon monoxide, in the lungs or elsewhere.

In these experiments Killick observed no increase in hemoglobin or in the number of red cells, but it must be remembered that exposures to carbon monoxide were not very frequent.

There is some evidence that acclimatization in monkeys may be of

the same type as that described by Killick in the human subject. Van Bogaert, Dallemagne and Wegria (1938) exposed a monkey (Macacus rhesus) daily to a concentration of carbon monoxide sufficient to produce 30 per cent saturation of its blood in the course of an hour. The actual degree of saturation produced varied somewhat from day to day, probably as a result of the varying activity of the animal, but it oscillated about 30 per cent during the 6 months of the experiment. At first the atmospheric concentration necessary to give 30 per cent saturation was 0.01 per cent carbon monoxide, but in the course of six months the concentration had to be raised gradually to 0.15 per cent carbon monoxide in order to maintain a blood saturation of about 30 per cent. It is interesting that during this experiment neither the oxygen capacity nor the red cell count of the blood altered appreciably.

There is considerable clinical evidence of an increase in hemoglobin and red cell count in the blood of men whose work involves regular or frequent exposure to carbon monoxide (Karasek, 1909–1912; Beck, 1927, 1936; Jenkins, 1932). Red cell counts as high as 9.68 millions have been recorded (Karasek) but usually the hemoglobin index does not show as great a rise. Anemia is also recorded as resulting from chronic poisoning (Beck and Fort, 1924-25; Biedermann, 1938), but it seems possible that in such cases there has been exposure to other toxic gases in addition to carbon monoxide. In the same way the slight increase in fragility of the red cells that has been attributed to carbon monoxide (Williams and Smith, 1935) is probably caused by some other constituent of illuminating gas (Mayers, Rivkin, Krasnow, 1930).

It appears that this increase in the oxygen carrying capacity of the blood, associated with acclimatization in man as in animals, may be supplemented, under certain conditions, by the change in the degree of saturation of the blood already described, so that the blood of the acclimatized individual attains a lower percentage saturation than that of the unacclimatized, when both individuals are exposed to the same atmospheric concentration of carbon monoxide.

In spite of the development of acclimatization there is little doubt that chronic exposure to low concentrations of carbon monoxide does cause ill-health. Reference has already been made to some of the symptoms of this condition, and the commoner symptoms may be listed as follows: Headache, dizziness, drowsiness, digestive disturbances, dyspnea, palpitation, muscular weakness (Mayers, 1930; Beck, 1936).

Treatment. The treatment of acute carbon monoxide poisoning is directed toward shortening the period of anoxemia by hastening the

elimination of carbon monoxide from the blood. Hill and Flack (1908) were the first to suggest the use of carbon dioxide for this purpose, and Henderson and Haggard (1920 and 1922) demonstrated the efficiency of carbon dioxide and oxygen mixtures by experimental work on dogs and later in a series of human poisoning cases. Sayers and Yant (1923) found that the rate of elimination of carbon monoxide from the blood was represented by the equation:

$$\text{Log}\, S' = \log S + (b \log e)\, t$$

where S was the initial saturation, S' the saturation after time t, and $(b \log e)$ was a constant; they found that the value of this constant varied with the treatment employed, the rate of elimination being fastest with a mixture of about 10 per cent carbon dioxide in oxygen, slowest with air, and intermediate when pure oxygen was administered. Stadie and Martin (1925) in their experiments on dogs obtained curves for the rate of elimination of carbon monoxide which conformed to the above equation, and they also showed that the effect of carbon dioxide in lowering the pH of the blood was an important factor in its action.

It appears from Henderson and Haggard's work (1922), however, that this equation does not represent the rate of elimination in the early stages of recovery from severe anoxemia; in these cases the breathing is at first depressed, and in the absence of treatment elimination is very slow. This additional factor emphasizes the importance of administering carbon dioxide to stimulate the breathing.

Various additional forms of treatment have been recommended from time to time, but most of them are useless, if not actually harmful; e.g., blood transfusion, bleeding, injection of methylene blue. The latter suggestion (Barron et al., 1928a, b; 1930, 1932; Brooks, 1932) is based on the effect of methylene blue in stimulating oxygen consumption independently of the tissue enzyme systems, but Haggard and Greenberg (1933) and Nadler, Green and Rosenbaum (1934) have pointed out that methylene blue converts hemoglobin into methemoglobin, and therefore must increase the degree of anoxemia. Haggard and Greenberg in experiments on animals demonstrated methylene blue to be useless or even dangerous in carbon monoxide anoxemia. Henderson and Haggard (1922) consider that if oxygen and carbon dioxide are administered, together with artificial respiration if this is necessary, the less the patient is disturbed by other forms of treatment the greater his chances of complete recovery. The only effective treatment of chronic carbon monoxide anoxemia is to put an end to the exposure to the gas.

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FLUCTUATIONS IN BODY IODINE

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Since the discovery of iodine by Courtois, 1811, (28), two events stand out in our unfolding knowledge of iodine in relation to physiological processes. These are the discovery of iodine in the thyroid by Baumann, 1895, (9), and the isolation of crystalline thyroxine by Kendall, 1914 (84). Yet in themselves these discoveries contributed little to the understanding of biological mechanisms. They are supremely important because they serve as jumping-off points for painstaking researches by scores of other students who, bit by bit, have brought our knowledge to its present pitch.

The chief impediment to these studies has been a lack of suitable techniques and, in particular, of analytical methods. This continues to be true, largely because of the very low concentrations of iodine found in most biological systems. Indeed, the major difficulty in reviewing work on the physiology of iodine lies in the evaluation of technical procedures. This point is evident from a study of classical monographs on the subject, such as those of Kendall (85), von Fellenberg (55), Scharrer (141), Harington (69), Elmer (36), Leipert (94) and McClendon (115). To these sources the present article owes much.

In 1850 Rabourdin (132) published his method for determining iodine, and forthwith there began a series of investigations which can be traced through Chatin (19) and von Fellenberg (55) to the present day. Although Bubnow (15) identified the thyroid colloid as protein in nature, it remained for Baumann (9) and Hutchison (81) to study thyroglobulin as an *iodo*protein. The identification of 3,5 di-iodo-l-tyrosine by Wheeler and Jamieson (165) established a working hypothesis for the protein's constitution which led to its isolation by Harington and Randall (72) many years later (1929). Meantime, Harington and Barger (71) established that thyroxine also is a derivative of tyrosine. Finally, in the last decade it has become clear that the thyroid hormone

is stored in a characteristic colloid form, containing at least two iodo-amino-acids in peptide linkage (Harington and Salter, 73).

In recent years attention has turned to the measurement of the minute amounts of iodine in the blood and in various tissues. These methods are still crude, considering their objective, but they have been developed to the point of dividing this iodine into characteristic fractions, i.e., "organic" and "inorganic." Recent discussions of such technique have been given by Elmer (36) and by McClendon (115). Amounts as low as 0.5 gamma (0.000,000,5 gram) can be measured within an error of 0.1 gamma, an accuracy satisfactory enough for many physiological problems. These procedures, however, demand a considerable analytical skill. When several microchemists analyze the same sample of blood and report values for iodine ranging from 7 to 70 micrograms per cent (135), it is clear that technique is still a major problem.

Fortunately, despite these technical difficulties, new improved methods are appearing which yield consistent results in different laboratories; and the fantastically high values for blood and urinary iodine concentrations, so common a decade ago, are rapidly disappearing. Consequently, it is possible already to report much information which is of permanent physiological interest. Simultaneously, other data dealing with pharmacological observations and with clinical conditions have accumulated in even greater volume. Although the iatrochemist has much to teach the physiologist about iodine metabolism, such studies fall perforce outside our present limitations.

I. Iodine Stores in Body Tissues. Justus (83) decided that all cells contain iodine, and in recent years reliable quantitative evidence has been adduced in favor of his point of view. Nevertheless, the iodine content of the whole human adult is small; it varies between 20 and 50 mgm. (154), (36, p. 84). (This amount is equivalent to less than 10 minims of liquor iodi compositus, U. S. P.) Of this whole quantity, the muscles contain one-half, the skin one-tenth and the skeleton one-seventeenth.

The total iodine in the blood of normal, fasting individuals is probably less than 10 gamma per cent, and contributes less than one-tenth of the whole iodine in the body. The human thyroid normally holds at least one-fifth of the total iodine, even though its mass is only one-five hundredth of the whole body. Thus the thyroid iodine (40,000 gamma per cent) is a thousand times as concentrated as muscle iodine (30 gamma per cent). Lower values for all tissues may be encountered

when the iodine supply is very low, but the normal thyroid maintains its capacity for the preferential trapping of iodine even when the amount of ingested iodine is greatly reduced.

The next highest iodine concentrations are found in other glands of internal secretion (20). These include the anterior pituitary (36, p. 86) (37), epiphysis, ovary, adrenal cortex (47) (36, p. 85) and parathyroid. In general, the iodine concentration of these endocrine organs is some fourfold that of other body tissues, which contain only about 20 to 30 gamma per cent. The increased concentration in the endocrines largely disappears after thyroidectomy (154) (36, p. 87), whereas the iodine in skeletal muscle and other tissues remains relatively undisturbed. This fact raises the possibility that iodine in these endocrine glands is of thyroidal origin, a question to be studied further.

Types of iodine compound in the organism. At the present writing, tissue iodine usually is classified as *1*, inorganic iodide, and *2*, organically bound iodine, which may be *a*, like thyroxine, insoluble in dilute aqueous acid, or *b*, like diiodotyrosine, relatively soluble in dilute aqueous acid.

Iodide. It is clear that most body fluids contain inorganic iodide, the concentration of which (roughly, 2 gamma per cent) will be discussed later. Likewise, red cells are known to contain inorganic iodide. These concentrations, it will be noted, are rather low under ordinary physiological conditions. They may possibly be increased up to tenfold in hyperthyroidism, and certainly after the therapeutic administration of iodide up to an hundredfold (see below, section II, p. 358).

Pincussen and Roman (131) studied the electrodialyzable iodine in the mixed tissues of white mice. The average iodine was 3.4 mgm. per cent, of which the ratio of organic to presumably ionized iodine was 1.85. Such figures for iodide represent merely the total mass of tissue, and neglect the possibility that some of the diffusible iodine might not be iodide; they represent, therefore, maximal possible values. In the thyroid itself, relatively little inorganic iodide is found, even after recent administration of iodide to the organism (69), when it may reach to 10 per cent of the total thyroidal iodine, only to disappear rapidly after the medication is assimilated.

Naturally Occurring Organic Iodine Compounds. The other chemical compounds which contain iodine are iodinated tyrosine and iodinated thyronine. When elementary iodine is fed, as in liquor iodi compositus, U. S. P., it soon is changed in the gastrointestinal tract either into organic iodide or, by combination with protein split-products, into

diiodotyrosine. Likewise, the iodine-poor human thyroid fixes circulating inorganic iodide in the form of diiodotyrosine soon after its administration (65). Recently Hertz, Roberts, Means and Evans (78) have studied the fixation of radioactive iodine and have found that the process may be nearly complete within 15 minutes after a single dose, given intravenously. Subsequently, iodine is found in the form of tetraiodothyronine (i.e., thyroxine). Whether the intermediary stage, diiodothyronine, is present in the thyroid gland is still unknown.

Studies of artificially iodinated proteins (11) (7) (108) suggest that other iodine compounds should be looked for in mammalian tissues. Indeed, monoiodotyrosine has been described (126) in hydrolysates of iodinated casein. Likewise, Bauer and Strauss (7) found evidence of iodohistidine (both the mono- and diiodo compounds). From the standpoint of chemical evolution, it should be noted that diiodotyrosine ("iodogorgonic acid") occurs in sponges and in "corals" (77). The origin of thyroxine in the evolutionary scale still remains to be studied carefully.

The Thyroid Reserve. The thyroid protein itself, iodothyroglobulin, is a composite molecule containing both diiodotyrosine and thyroxine in peptide combination as integral parts of the molecule (73). In laboratory preparations the size of its molecular aggregate has been determined by ultracentrifugation to be about 670,000 (75). Under special laboratory conditions, however, this aggregate may split into four particles (156), and there is no certainty as to its size in vivo. It seems highly probable, however, that its colloidal properties serve to anchor the hormone in its storage form, the "thyroid colloid" of the microscopist.

A detailed study of the composition of thyroglobulin, with especial reference to its component amino-acids, has been made recently by Cavett (18) and by Brand and Kassell (14). It is by no means clear yet that the protein is a chemical entity in the sense that the crystalbumin of blood plasma is a definite entity. The protein has never been crystallized. Furthermore, depending upon the supply of iodine available, the iodine content of thyroid protein may vary from 0 to 1 per cent (8). The relative proportion of thyroxine to diiodotyrosine also varies. This ratio tends to be low when the total iodine is low (102), as described in another section. In thyroglobulin prepared from the glands of Argentine sheep by the author, for example, the partially purified, heat-coagulated protein contained over 1.2 per cent iodine, of which over 60 per cent was apparent thyroxine. By contrast, average human thyroglobulin in Boston contained 0.22 per cent iodine, of which

only 25 per cent was thyroxine-like. Nevertheless, Brand and Kassell have demonstrated a systematic intramolecular arrangement of those amino-acids which do not contain iodine.

Although it has been suggested (63) that thyroxine is lightly bound to a colloid vehicle, it seems clear that l-thyroxine is liberated from firm union in the peptide chain by the action of proteolytic enzymes (73). In normal thyroglobulin, both the diiodotyrosine and the thyroxine are in the l-configuration (72) (73). It remains to be seen whether the protein of thyroid cancer contains the d-forms, as suggested by the work of Kögl and Erxleben (90). The latest evidence seems *not* to favor this possibility.

The Thyroid Colloid. By microdissection methods fresh thyroid follicles may be "teased" apart and from them the intrafollicular colloid may be withdrawn as a viscid, albuminous liquid (134). The crude colloid in the follicle is adulterated with varying amounts of nucleoprotein (74). This intrafollicular nucleoprotein, however, has no known physiological function and is thought to originate from disintegrating or secreting cells of the follicular wall. It will not be considered further in this review.[1]

It is not known definitely in what chemical form the hormone is released from the colloid stored in the follicle. By enzymic hydrolysis Harington and Salter (73) obtained a peptide preparation of thyroxine which was assayed in human myxedema by Salter, Lerman and Means (138). Its greater solubility suggested that it might resemble the true circulating hormone. Further assays suggested that the remaining non-thyroxine iodine in thyroid protein also contributed to its potency in human myxedema. Harington (70) summarized this paradox by suggesting that the active thyroid secretion consisted of a peptide chain containing both thyroxine and other amino-acids, including diiodotyrosine. This problem is interesting from a therapeutic standpoint and will be discussed further in section III.

Other Tissues. Not much is known about the state of the organically bound iodine in other tissues. There is a thyroxine-like fraction, "T," in the blood (43), and probably also a diiodotyrosine-like fraction, "D" (161). Neither of these fractions is supported by a positive chemical identification, although the "T" fraction has been confirmed by bio-assay (168).

Direct attempts to isolate thyroxine from tissues at the present time

[1] In addition, Williamson, Pearse and Cunnington (166) have adduced evidence of an inactive thyroid "secretion," nearly free of iodine, found in tissue showing "hyperplasia."

are complicated not merely by difficulties in technical manipulation, but also by a more fundamental and, at present, surprising difficulty. This complication may be summarized as follows:

In recent years it has been found that such a nondescript protein as casein, when iodinated and subjected to alkaline hydrolysis, yields compounds related to thyroxine (1) (108) (136). Indeed, even unhydrolyzed serum albumin, after iodination, relieves human myxedema in classical fashion (103). The method of isolation involved in treating these nondescript proteins is quite similar to that for isolating thyroxine from thyroid protein. Consequently, the recovery of thyroxine from any tissue might be merely the result of an artefact. An alternative interpretation would be that organically bound iodine in the body is always in thyroxine-like form, a supposition which is by no means suggested by the classical studies of artificial iodoproteins (150) (7).

Biological considerations, however, indicate obviously that some thyroxine derivative circulates from the thyroid to the tissues by way of the blood stream. Moreover, hormonal activity has been demonstrated in tissue. Thus Kommerell (91) fed muscle meat obtained from both normal and myxedematous animals. The latter flesh failed to increase oxygen consumption in test animals, although the normal flesh did so. Likewise, tissue extracts have been studied from the standpoint of their effect on metamorphosis. Also Zawadowsky and Perlmutter (170) have used the metamorphosis of axolotl to amblystoma to detect the presence of thyroid hormone. This biological phenomenon occurs completely with as small a dose of thyroxine as 10 gamma per 10 grams of body weight. Accordingly, when iodine concentration is rigidly controlled, the confusing effects of other iodine compounds can often be excluded. When this test is performed carefully, therefore, it is more specific than the Gudernatsch (64) tadpole method or Uhlenhuth's procedure (163), and offers greater hope for detecting thyroidal activity in tissue extracts or in body fluids. By this method, for example, Zawadowsky and Asimoff (169) were able to demonstrate increased hormonal activity of the liver, kidney and blood serum in guinea pigs which had received thyroid preparation about six hours previously.

Unfortunately, such attempts to estimate concentrations of hormone are at present in the pioneer stage. Obviously, improved micromethods for detecting thyroid hormone in blood and other tissues are badly needed.

Temporary iodine storage. When iodine is administered from an

external source, there results a temporary stage of metabolic flux which alters the characteristic concentrations just described. These alterations vary with the source of extra iodine.

Iodide. The fate of inorganic iodide after absorption depends in part on the dosage used and on the physiological state of the recipient. Maurer and Ducrue (114) gave rabbits 10 gamma iodine per kilogram in this form and analyzed various tissues both one day and four days later. They found a great increase in the thyroid iodine, corroborating the classical findings of Marine (111) in the iodine-hungry dog. The liver, skin and lungs showed very high iodine contents as temporary storage depots, but after four days only the heart, kidneys and lungs remained unusually rich in iodine.[2] Increased concentrations have been found temporarily also in the stomach and parotid (104). Obviously, the organism does not retain extra iodide long. With larger doses in guinea pigs, the thyroid stores only 4 per cent of the extra iodine accumulated, but the skin, hair, muscles and bones store iodine for many days at a high concentration (55). The lungs and trachea show very high concentrations temporarily, but soon lose the extra iodine despite the fact that the expired air shows no increase in iodine content (114).

Organic Iodine Compounds. Simple organic iodine compounds likewise accumulate in the skin (106). Furthermore, after three weeks' daily administration, iodotryptoflavin is stored, especially in the brain and kidneys (54). Experiments with diiodotyrosine are unsatisfactory because as yet there is no micromethod available for determination of traces of this compound.

The fate of thyroxine derivatives, however, has been studied both by iodine analysis and by the bio-assay method of Zawadowsky and Asimoff (169). Asimoff, Estrin and Miletzkaja (3) studied the effect of a single dose of "thyroidin" by mouth in dogs and roosters. They found that iodine accumulated in the thyroid, kidneys, liver, blood, pancreas, skin, heart and spleen. Likewise, increased iodine is found in the muscles and bones of the rat five days after a single intravenous injection of thyroxine (93). Small amounts of this iodine remain stored for a long time in the animals' tissues, especially in the liver, muscles, thyroid and skin (125). In another section the selective accumulation of thyroxine iodine by the hypophysis has been discussed (155). Whether the tuber cinereum shows this in even more marked degree (148) has not yet been decided.

[2] The distribution of this iodide throughout body fluids has been studied by Wallace and Brodie (145).

The biological method of Zawadowsky has yielded interesting information as to the fate of thyroxine derivatives in bodily economy. Zawadowsky and Asimoff (169) fed "thyroidin" to mammals and birds and estimated its concentration in various tissues by bio-assay. During the first few hours the hormone is trapped in the liver to a large extent. Thereafter it increases in the blood plasma and in the kidneys. After 24 hours the hormone largely disappears. Birds appear to destroy the hormone less rapidly than mammals.

II. The Circulating Iodine. Iodine in respired air. The atmosphere of lowlands near the sea may contain 0.4 gamma iodine per liter, so that a man conceivably might inhale from 3 to 5 gamma daily. Consequently, it is difficult to estimate iodine truly excreted by respiration. By actual measurement a man can exhale 10 gamma in 24 hours, i.e., 25 per cent of the total excreted (143). Presumably this iodine is largely in the form of iodide. Szász (157) found as much as 24 to 33 gamma exhaled per day in a mountain atmosphere which was essentially iodine-free, but this larger amount may be related to the large water excretion by the lungs at high atmospheres.

Iodine in cerebrospinal fluid and in lymph. The nature of the iodine in cerebrospinal fluid is still unsettled. Its concentration is lower than that of whole blood. The following pairs of comparative values are given in gamma per cent for blood and spinal fluid, respectively: 10.6 and 7.4 (68); 13 and 10 (80); 7.7 and 2 (117). It is likely that the cerebrospinal fluid contains no thyroid hormone (117), but further study is needed.

The lymph contains an appreciable quantity of iodine (149), but the mechanism which governs its concentration remains obscure. The puzzling fact is that dogs whose *blood* iodine is from 10 to 18 gamma per cent yield thoracic duct *lymph* which contains 42 gamma per cent. Furthermore, when the animals are fed plenty of milk, values of from 290 to 378 gamma per cent are found. Together, these findings suggest that the high concentration in the thoracic duct is due to contributions of iodine from the intestine and the liver.

Iodine in milk. In the normal lactating animal, considerable quantities of iodine are secreted by the breast (27). In the first day of the human puerperium the colostrum yields only 2 to 5 gamma iodine in 24 hours, although the iodine concentration ranges from 8 to 45 gamma per cent (36, p. 105). When lactation is established, from 20 to 47 gamma iodine may appear in 24 hours in a concentration of 3 to 12 gamma per cent (95). Thus the monthly excretion of iodine amounts

to from 2.5 to 3.2 mgm. (162). The chemical status of this iodine is not clear. The major part of milk iodine is attached to organic material either by chemical combination or possibly by adsorption (55). Studies of lactating cattle have shown that iodine in the milk varies with the season and with the type of fodder. Seacoast pasturage increases the milk iodine (142). There is much less iodine in butter fat than in skimmed milk (116). The nursing infant does not receive thyroid hormone by way of the milk (35), nor does the milk yield a thyroxine-like fraction after hydrolysis (45) (44).

Iodine in sweat and saliva. Iodine is excreted by the skin in variable amounts; data are not available on variations in concentration. In profuse sweating the total in sweat may be over 30 per cent of the total iodine excreted (55). On the other hand, Szász (157) found practically no cutaneous excretion in the mountains at 1000 meters' elevation.

The concentration of iodine in saliva may vary from 0 to 362 gamma per cent (149), but the total amounts excreted by this channel are small. After the therapeutic administration of iodine, high concentrations appear in the saliva within a few minutes (32).

Blood iodine. *Physiological Range.* It has been clear, at least since the work of Kendall and Richardson (87), that normal blood contains iodine in a characteristic concentration. Normally, this amount is rarely less than 3 gamma per cent or more than 20 gamma per cent, as recorded by the majority of methods now available (36, p. 91). The blood of various animals is similar to that of man. Of course, the concentration is subject to fluctuation. Thus the level is increased somewhat by an iodine-rich diet; indeed, therapeutic doses of iodides (e.g., 1 gram of sodium iodide) may raise the level temporarily to several hundred gamma per cent. Likewise, a diet of iodine-rich foods, especially marine fish and seaweeds, increases the blood level. Sturm and Buchholz (154), among others, have found that seasonal variations in blood iodine occur parallel with changes in thyroid iodine. Löhr (107) failed to confirm this, but it should be remembered that in certain localities (e.g., the British Isles) thyroid iodine may not be subject to remarkable fluctuation (113). The blood iodine may rise at the time of the climacteric in women (16), but this phenomenon is not constant.

There is considerable diversity of opinion about the distribution of iodine between red cells and plasma. It seems clear that the plasma contains the major portion. Representative figures indicate about 8

to 12 gamma per cent in the serum and only 4 to 6 gamma per cent in the red cells (62). Trevorrow (161) studied a series of analyses of whole blood and plasma and found the total iodine to be distributed in proportion to the water content of the plasma and cells. This statement should be considered as a first approximation.

QUALITATIVE NATURE OF BLOOD IODINE COMPOUNDS. Ironically enough, despite many quantitative studies of the partition of the blood iodine, the nature of these fractions is largely unknown. Various methods have been used to fractionate the blood, but they are so arbitrary that it is difficult to compare results obtained by one method with those by another procedure. A systematic study of the blood iodine by rigorous physicochemical methods is sorely needed. Meanwhile, it is possible to draw tentative conclusions only.

Even if the plasma and red cells contain iodine in equivalent concentration in terms of water (161), there is no evidence that these concentrations necessarily represent the same chemical compounds. Indeed, Zawadowsky and Asimoff (169) found that, on feeding thyroid, the plasma became biologically active, but NOT so the red cells. It will be important to learn whether red cells contain hormone because, if not, the plasma should be analyzed separately, in view of its greater physiological significance.

There are many "normal fasting" values for "inorganic" iodide in the literature, but it is dubious whether much of the normal blood iodine is truly inorganic unless iodide has been ingested recently (161). Ultrafiltrates of plasma from fasting animals contain little iodine unless potassium iodide has been fed to the animal recently. On the other hand, iodide added to plasma can be recovered quantitatively in the ultrafiltrate. When animals are fed iodides, however, the extra inorganic iodide is distributed between plasma and cells in (roughly) equivalent concentration, i.e., in terms of the respective water contents of the two phases (114). Although these filtration experiments indicate that most of the normal blood iodine is bound by protein, Trevorrow (161) has found that it can be removed from the protein almost completely by extraction with butyl alcohol or by continuous extraction with ethyl alcohol or acetone. In other words, the union with protein may be due to adsorption rather than to firm chemical combination. Thus by varying the procedure, the normal fasting blood iodine can either *a*, be left almost entirely adherent to protein, or *b*, be removed almost completely from protein. Unfortunately, many of the procedures employed in the past 15 years represent intermediate stages.

Thus the classical procedure of Lunde, Closs and Pedersen (110) for the determination of "organische" iodine gave the fraction which happened to be bound to protein after *one* extraction with alcohol. Similarly, McClendon (115) recently has used acetone to find the "hormone iodine" left bound to protein, despite the finding by Eufinger and Schulte (52) that all of the iodine can be removed by acetone if further extraction be made. In other words, it is hazardous to assume that alcohol-soluble (or acetone-soluble) iodine represents "inorganic" iodide, whereas the alcohol-insoluble (or acetone-insoluble) represents "organic" or "hormone" iodine alone. Similarly, the use of dialysis or electrodialysis (10) gives values for diffusible "iodide" which depend upon changing concentrations and rates of diffusion.

Recent analytical studies (161) (127) indicate that thyroxine, diiodotyrosine, thyroxine-proteose and thyroglobulin are all nearly completely precipitated with the proteins of blood by such agents as heat and acetic acid or zinc sulfate and sodium hydroxide. On the other hand, added iodide escapes precipitation. Such observations suggest that the normal fasting blood iodide concentration is only about 2 gamma per cent. Contrast this figure with the high values for alcohol-soluble "iodide" now in the literature! The author suggests that non-precipitable iodine be regarded henceforth as an index of inorganic iodide.

The protein-bound iodine of the plasma also may prove to be a useful measurement provided care is taken to obtain the maximal value possible. This figure would include the various constituents of thyroglobulin mentioned above, if present, and possibly other unknown iodine-containing substances which can be adsorbed on protein molecules. Indeed, when thyroxine is added to blood it may appear either in the "inorganic" or the "organic" fraction, depending upon the procedure employed (128). This fact may explain why Leipert (96) was able to fractionate diffusible iodine into fractions precipitable by silver and not so precipitable. Lipoid-bound iodine also has been suggested (164), but it seems insignificant if present at all (96).

Trevorrow (161) has been able to separate the butyl alcohol extract of blood iodine into thyroxine-like fractions and diiodotyrosine-like fractions. She concludes that "the greater part of the blood iodine possesses properties similar to those of thyroxine and diiodotyrosine, and a portion of this is not diiodotyrosine but is thyroxine-like in its solubility."

In this connection, immunological observations recently made by Lerman are illuminating. He produced rabbit antiserum "which would

detect human thyroglobulin in concentrations as low as 0.15 to 0.3 mgm. per cent, i.e., in a concentration equivalent to less than 1 microgram per cent of iodine. *This was true whether the thyroid protein was dissolved in physiological salt solution or in normal human serum.* He was unable to detect such a concentration in normal human serum, thyrotoxic serum (pre- or postoperatively), or in myxedema. An exception to this statement was the finding that after surgical manipulation of the thyroid, hormone can be detected by this means frequently in the thyroid veins during operation and occasionally in the systemic venous blood for as long as 24 hours after operation. He was unable to detect any thyroglobulin in human urine. One preparation of the antiserum, which was unusually potent, was capable of disclosing a concentration of thyroglobulin as low as 0.05 mgm. per cent. With this potent antiserum Lerman was unable to detect any thyroglobulin in serum from hyperthyroid patients (98). These observations suggest strongly that the circulating hormone differs from the storage form previously described in section I.

For the sake of uniformity in the literature, the author suggests that in clinical and metabolic studies the following program be followed: *1.* Plasma to be separated from red cells, at least until it appears that the iodine of red cells reflects precisely that of the plasma. *2.* The plasma iodine to be separated into "I" iodine, presumably iodide because ultrafilterable or non-precipitable, and into "P" iodine, the maximum adsorbable on protein or precipitable with protein molecules. *3.* The "P" iodine to be separated, according to Leland and Foster (97), into a "T," thyroxine-like fraction and "D," diiodotyrosine-like fraction. This plan will admit of direct comparison of concentrations in the blood with those in the thyroid gland itself, or with pharmaceutical preparations and derivatives of thyroglobulin or of iodoprotein (102) (103). Indeed, in one such experiment with beef plasma, Trevorrow found 92 per cent of the total iodine in the "P" fraction, of which one-third was "T" iodine and two-thirds "D" iodine. These are the values one would have expected from the normal human thyroid in Boston!

After human blood has been hydrolyzed directly in alkali, it yields both "T" and "D" fractions (43). Although the hydrolysis admittedly destroys some 20 per cent of added thyroxine, nevertheless it increases qualitatively the reliability of the results. The "T" iodine is between 2.9 and 4.8 gamma per cent, i.e., about one-third of the average total iodine (12 gamma per cent). Thus 5 liters of human blood would

contain nearly 0.5 mgm. of apparent thyroxine; 25 liters of horse blood, 1 mgm.; and the entire blood of a dog 0.05 to 0.025 mgm. of apparent thyroxine.

Extant Clinical Data. These academic considerations notwithstanding, there exists a large mass of clinical and metabolic material based on the concept of "anorganische" and "organische" iodine as separated by the alcohol precipitation of Lunde and Closs (109) or by similar procedures. Because these data convey important information, we must review them carefully, remembering that "anorganische" iodine often contains little iodide and that "organische" iodine represents only a portion of the maximal iodine which is adsorbable on protein. The "organische" iodine is sometimes called "hormone iodine" (115). The consistent behavior of this value in normal and in hyperthyroid individuals offers some hope that eventually this arbitrary fraction may be closely identified with the "T" fraction described above, even though it probably constitutes the net effect of several compensatory errors.

Fasting Blood Iodine. Because of the technical difficulties encountered in separating small amounts of iodine, not to mention the diversity of analytical techniques in vogue at present, comparative values obtained by the same investigators are more reliable than absolute values obtained by different procedures in various localities. Obviously, when stating blood iodine in studies of pathological physiology, each investigator should make clear his range for normal values.

As regards whole blood iodine, fasting values obtained by Gutzeit and Parade (67) (67) in Breslau are instructive:

For normal men: 9 to 16 gamma per cent (average 14 gamma per cent)
For normal women: 11 to 20 gamma per cent (average 17 gamma per cent)
"Organische" iodine: 3 to 5 gamma per cent

$$\text{Normal "iodine quotient"} = \frac{\text{"organische"}}{\text{"anorganische"}},\ 0.2 \text{ to } 0.5$$

In Boston, Perkin, Lahey and Cattell (129) found in normal adults an average of 6.6 gamma per cent, ranging from 1 to 10 gamma per cent. In Ohio, Davis, Curtis and Cole (33) found in normàl adults an average of 10.1 gamma per cent, ranging from 8.5 to 16.2 gamma per cent. In children in New York City, Fashena (53) found average values, without sex difference: for infants less than 24 hours old 4.7 ± 0.33 gamma per cent, ranging from 1.0 to 11.0 gamma per cent; for others up to 13 years, 6.6 ± 0.15 gamma per cent, ranging from 3.0 to 12.0 gamma per cent.

Effect of Exercise. In studying blood iodine, it must be remembered that vigorous muscular activity causes an increase in the fasting blood iodine within a few minutes. There is disagreement about the extent to which the iodine rises; McCullagh and McCullagh (118) report a rise of about 9 gamma per cent, whereas Herxheimer, Mislowitzer and Stanoyéwitch (79) found increases of over 150 gamma per cent. The normal level is not restored for over two hours after cessation of the work.

Circulating Iodine after Administration of Iodine Compounds. The total blood iodine concentration at a given moment represents the net result of many complicated processes. Among these are the quantity and type of compound used, the rate of intestinal peristalsis (i.e., presence of diarrhea), the ease and rate of intestinal absorption or of parenteral injection, the avidity of the thyroid for new iodine, the permeability of various tissue cells, the state of kidney function and many other factors. Furthermore, different species of animal react differently. Consequently, manifold variations are possible and it is possible to consider only certain typical combinations of circumstances.

Iodide Administration. Potassium iodide is so rapidly absorbed that the blood iodine concentration rises to a peak within 30 minutes if doses of 2 grams or more are ingested (154). Under these circumstances, the blood iodine concentration may rise well above 1000 gamma per cent. With smaller doses (e.g., 0.5 gram) the peak is not reached until 90 minutes (164). Then the blood iodine falls, to reach the initial level in 48 hours. Thus the bulk of the iodine appears rapidly in the circulation and again disappears rapidly (38). Elsewhere (171), iodine tolerance curves are discussed to show that the functional activity of the thyroid regulates this fluctuation in blood iodine to a large extent. Eventually a large part of the extra iodine is eliminated through the kidney. A much smaller amount is eliminated into the intestine through the bile and intestinal secretions. Possibly the stomach also eliminates important amounts of iodine (104), thus adding to the intestinal content.

After intravenous injections of iodine, the blood level drops rapidly from its initial peak. The type of curve obtained, however, is determined largely by the dose injected. Elmer (36, p. 155) has injected small doses (1.7 mgm. potassium iodide) and found that in normal individuals the blood iodine returns to control levels in six hours. Also Perkin and Lahey (130), using larger doses (i.e., 0.6 gram by mouth), found a more protracted hyperiodemia, as did Fitz (57).

Even though the total blood iodine rises, the *"organische"* moiety thereof falls (164). Thus, after the oral administration of 0.5 gram of potassium iodide the "organische" moiety may drop from 10 gamma per cent to 3 gamma per cent when the total iodine approaches its peak. Later, the total iodine approaches the normal after 48 hours, as does the "organische" moiety. Thus the curves for total and "organische" iodine, respectively, at first separate and then approach each other again. It may be that the reason for the drop in "organische" iodine is suppression of the thyroid secretion (152) (110). If true, the rapidity of the fall in concentration is remarkable because thyroid changes are usually considered sluggish.

Administration of Thyroxine Derivatives. The blood iodine curve following the administration of whole thyroid or of thyroglobulin has not been studied well. Veil and Sturm (164), however, studied the effect of feeding small doses of a desiccated preparation called "thyroidin" (containing 150 gamma iodine). In two hours the peak was reached at a questionable 41 gamma per cent, and in 24 hours the blood was normal. More studies are needed on this point. Both "organische" and "anorganische" fractions increase, suggesting that decomposition of the hormone occurs in the gastrointestinal tract, in the liver (49), or elsewhere. Nevertheless, after the enteral administration of thyroglobulin some antigen (presumably thyroid protein) is found in the blood stream (76). Most of the ingested thyroglobulin is split, however, as expected, into peptones or peptides in the gastrointestinal tract (6) (5).

Thyroxine per Os. When thyroxine is administered by mouth appropriately dissolved in alkali,[3] the blood iodine of dogs reaches 28 gamma per cent in two hours and falls to normal in 12 hours (146). After injecting 2 mgm. of thyroxine, Böe and Elmer (12) found that there was an almost immediate fall from the initial peak of 36 gamma per cent. In 24 hours the blood was again normal. It is highly significant that the rise was entirely in the "organische" moiety.

Relation of Blood Iodine to Basal Metabolic Rate. It is generally stated that the blood iodine level does not run closely parallel to the severity of the disease. Nevertheless, Möbius and Nolte (124) found a certain correlation between the increase in basal metabolic rate and the increase in blood-iodine concentration. The author suggests that the correlation might be greater if the logarithm of the plasma con-

[3] Thompson and his collaborators (159) have shown that about 80 per cent of the drug is assimilated, when administered in this fashion.

centration be studied. He has compiled in such fashion the data of Elmer and Scheps (50), who stated that they failed to find an exact parallelism. These data show a considerable fidelity to the equation:

$$\text{B. M. R.} = \log (\text{Blood iodine minus } 3)$$

when blood iodine is expressed as micrograms per 100 cc. This relationship is shown in figure 1. The coefficient of correlation $r = 0.75 \pm 0.07$ and P is less than 0.01; i.e., the chances that this correlation is significant are better than 100 to 1.

When the "organische" moiety of the blood iodine is investigated, it is generally found to be increased more consistently than the total iodine (164). Gutzeit and Parade (66) have tabulated the ratio of "organische" to "anorganische" iodine. The normal quotient is 0.2 to 0.5, whereas in severe hyperthyroidism it may approach 4.0. Sometimes, however, the basal metabolic rate is found to be markedly increased with a fairly low "organische" iodine fraction (39). Likewise, the inverse combination may occur (34). Various factors conspire to obscure this relationship, viz., inaccuracy of present analytical methods, the possible presence of inactive organic iodine compounds (possibly degradation products of the hormone) and the difficulty of attaining in these patients a "steady state," physiologically speaking.

Gutzeit and Parade (67) (66) have laid great stress upon the behavior of the "organische" iodine and of their "iodine quotient," i.e., "organische"/"anorganische" fraction. In severe hyperthyroidism, these measurements indicate that the gland is working at maximal capacity, as indicated by the response to exercise. In normal persons, within an hour or two after vigorous exercise the "organische" iodine rises, and likewise the iodine quotient, to return to normal after 24 hours. In hyperthyroidism, on the contrary, there is no further rise and there may even be a fall in these values.

Elmer, Rychlik and Scheps (46) attempted to simplify the problem by determining the level of "thyroxine-like" iodine substances in the blood after hydrolysis. They used the method of Leland and Foster, slightly modified. In three cases studied the blood contained from 9 to 16 gamma per cent of "thyroxine" iodine, i.e., 50 to 60 per cent of the "organische" iodine or 27 to 44 per cent of the total iodine. Apparently a thyrotoxic man has from 0.8 to 1.4 mgm. of thyroxine in his blood, as compared with 0.4 to 0.5 mgm. in the normal person. More recently, Trevorrow (161) has improved the analytical technique so that, as data continue to accumulate, it may be possible to demonstrate a closer

parallelism between the basal metabolic rate and the concentration of presumable hormone or some mathematical function of it.

III. Thyroid Activity. Although the thyroid is the chief regulator of iodine metabolism, it is not the purpose of this review to discuss the

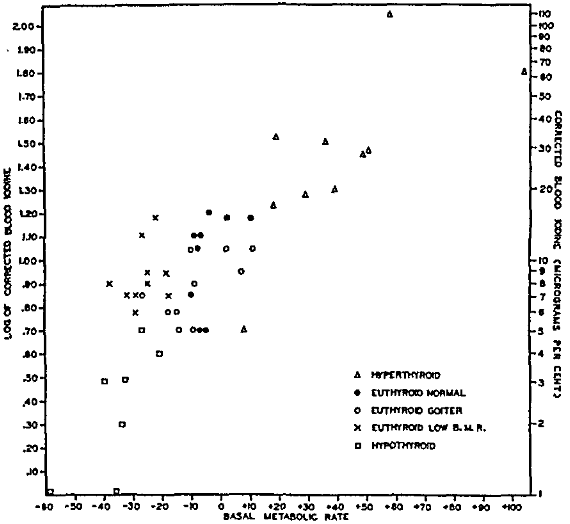

Fig. 1. Blood iodine and basal metabolism. Data for blood iodine concentration in several states of thyroid function, collected by Elmer and Scheps in Lwów. An arbitrary correction of 3 gamma per cent for inorganic iodide has been subtracted from the total blood iodine. The scale showing iodine concentration is logarithmic. The high correlation with basal metabolic rate leads to the inference that the two are related if 1, clinical classification is adequate; 2, analytical technique good; 3, exogenous iodide intake is low, and 4, a steady physiological state is attained before analysis is made.

thyroid gland, its chemistry and physiology (69). It must suffice here to consider certain quantitative aspects of its endocrine product, especially as it concerns iodine compounds and their calorigenic effect.

Manufacture of hormone. When elementary iodine or iodates

are fed, they are converted into iodide before absorption (22). Both iodine and diiodotyrosine can be absorbed from the intestine into the blood, and both can be removed from solutions perfusing the isolated thyroid gland. Although the gland may receive iodine from tincture of iodine painted on the skin or buccal mucosa (160) or by inhalation of ethyl iodide (99), nevertheless the usual source for hormonal synthesis evidently is iodide in food or medication. Using radioactive iodide, Hertz, Roberts, Means and Evans (78) recently have shown that the normal gland is surfeited with newly administered iodine within 15 minutes after administration. The newly trapped iodine is combined progressively with the thyroid protein, or else it diffuses back into the blood, so that after a day or two little inorganic iodide remains in the gland. Ultimately, only a small fraction of an ordinary therapeutic dose remains in the normal gland, although an iodine-starved gland may retain as much as 18 per cent of a single therapeutic dose (111). With repeated doses the iodine content of the gland may be raised to over 1 per cent, dry weight. After such treatment, for example, the thyroids of guinea pigs contained 57 mgm. of iodine, as compared with 15 mgm. in thyroids of control animals (114). Of course, organically bound iodine can also contribute to thyroid stores. For example, Klein, Pfeiffer and Hermann (89) administered "jodtropon" for a week to hemithyroidectomized animals. Of 22.6 mgm. of iodine so given, 5.1 mgm. were retained, of which 1.9 mgm. were recovered from the single thyroid lobe and 3.2 mgm. from all other tissues.

The total iodine in the whole thyroid of apparently normal adult men may vary from 2 to 28 mgm. of iodine (112). In fresh tissue the iodine concentration varies from 1.1 to 166 mgm. per cent (or 12 to 431 mgm. per cent in desiccated thyroid). Marine believes, however, that human tissue outside of the limits 0.1 to 0.55 per cent (by *dry weight*) should be considered abnormal. The difficulty in assessing normal values is enhanced by the fact that the iodine content varies with diet, topography, season, endocrinological balance and age. Furthermore, if iodine intake is low, not only is the content of the thyroid low; but compensatory hyperplasia of the tissue further reduces the gross iodine concentration and exaggerates the deficiency. Of the iodine so stored, normally about one-third is in "thyroxine-like" form in human glands, although in certain animals (e.g., Argentine sheep) this value may approach two-thirds. In other words, the major portion of the iodine incorporated in normal human thyroid protein has reached

the diiodotyrosine stage, but has not been built up into the more complex thyroxine.[4] When the gland is secreting hormone at full capacity, its total iodine reserve may be depleted to less than one-tenth of the normal average. Furthermore, its thyroxine-like reserve may nearly disappear, presumably because it is removed as fast as it is synthesized.

Various estimates of apparent thyroxine in the whole human thyroid indicate consistently a store of from 3 to 5 mgm. (37), from 0.2 to 9 mgm. (97) or from 0.9 to 7 mgm. (65). In dog thyroids, Elmer (37) found 0.7 to 1.1 mgm. of apparent thyroxine, in rabbits only 3 to 5 gamma. The corresponding figures for diiodotyrosine are 2.0 to 6.1 mgm. iodine in human thyroids, 0.2 to 1.19 mgm. in dog thyroids and 4 to 18 gamma in rabbit thyroids. These latter figures include inorganic iodide, which is presumed to be negligible.

Ebb and Flow of Thyroid Iodine. The gland can regulate iodine metabolism either by fixing iodine and anchoring it to colloidal molecules, or by releasing such iodinated molecules or fragments thereof into the circulation. The action of the gland depends largely upon the properties of the blood which perfuses it. Foot, Baker and Carrel (58) have studied isolated human thyroids preserved in the Lindbergh apparatus, and found that the final histologic picture depended on the nature of the perfusate, *not* on the previous condition of the gland. Likewise, Sturm (152) was able to study the result of various concentration gradients on the storage of thyroid iodine by working with isolated thyroids perfused with solutions containing various concentrations of iodide. Up to 500 gamma per cent of iodide in the perfusing fluid, the gland removes iodide from the arterial blood, thus producing a demonstrably lower venous concentration. Above a concentration of 500 or 600 gamma per cent, a short period of iodide retention is followed within 20 minutes by iodine elimination, so that the iodine level in venous blood is higher than in the arterial blood. Thus the gland's iodine reserve is reduced and organically bound iodine is released. This latter situation seems highly abnormal, and probably has little direct physiological significance, but it does demonstrate that the storage mechanism is reversible. It is puzzling that organic preparations like "jodtropon" or even thyroxine, when perfused through the isolated gland, are not themselves stored. Nor do they influence retention or release of thyroid stores (152). Because this finding might

[4] It is conceivable that a very small amount (2 per cent) of the iodine may exist in lipoid combination (164).

suggest that iodine can enter the thyroid only in inorganic form, it deserves further study. Unfortunately, such data on diiodotyrosine are not available.

Sturm (152) also tested surviving thyroids from dogs which had been "satiated" with iodide for one or two weeks previously. These glands, when perfused with a solution containing iodide, quickly reacted by elimination of iodide! Three weeks after cessation of iodide feeding to the animals, the gland approached the normal reaction. Similar studies were made with glands from dogs which had been rendered thyrotoxic by administering "thyroidin" or thyroxine for several weeks. Such glands were atrophic, reduced to nearly half the normal size and showed flattened acinar epithelium lining follicles filled with colloid. Four such glands, however, reacted normally to perfusion with iodide by storing iodine. One other excreted iodine when the concentration in the perfusate reached 220 gamma per cent. Apparently the hormonal iodine, exogenous in origin, does not often yield enough iodide (by decomposition) to satiate the gland.

Daily Production of Hormone. On the basis of substitution therapy in human athyreosis, it seems clear that the human thyroid secretes about $\frac{1}{3}$ mgm. of thyroxine or its equivalent in 24 hours. On the basis of iodine balances, the optimal daily requirement is not less than 100 gamma iodine nor more than 200 gamma iodine (36, p. 277). Means (119) summarized his own data and the data of others, and reached much the same conclusion. Eppinger and Salter (51) confirmed his value by treating human myxedema with purified human thyroglobulin.

The amounts of hormonal iodine in the body can be estimated from the rate of decay of the metabolic rate in myxedema (13). Such calculations suggest that, exclusive of the thyroid, an average human adult contains some 10 to 20 mgm. of iodine, of which about two-thirds is in the form of thyroid hormone. These values are surprisingly consistent with actual analyses of tissues given elsewhere. The author and Lerman (100) have similarly observed the daily consumption of a single hyperthyroid individual at various levels of metabolism. This patient, previously reported by Means and Lerman (120), was subsequently treated alternately with crystalline racemic thyroxine (intravenously) and with Armour's desiccated thyroid (U.S.P.) by mouth. Taken together with *1*, the decay curve, and *2*, the iodine response reported by Means and Lerman (120), these additional data—i.e., on *3*, daily utilization at various levels; *4*, differential effect of thyroxine as compared with whole thyroid, and *5*, the theoretical hormone content of the

body tissues, as estimated by integration of the decay curve against time,—all collected on a single individual, are extremely interesting because they supplement and reinforce one another. Data on items

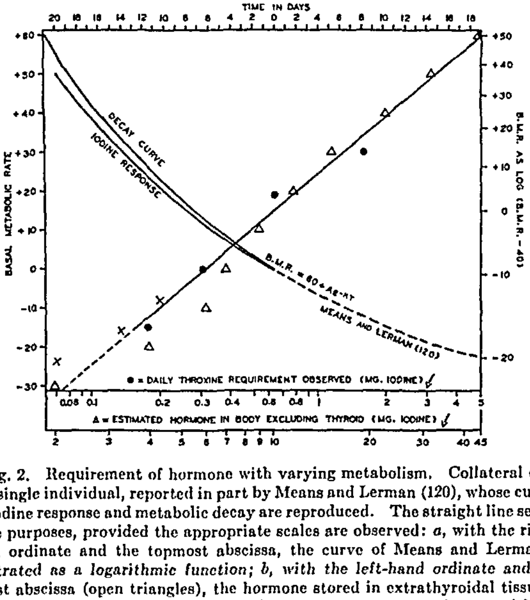

Fig. 2. Requirement of hormone with varying metabolism. Collateral data on a single individual, reported in part by Means and Lerman (120), whose curves for iodine response and metabolic decay are reproduced. The straight line serves three purposes, provided the appropriate scales are observed: *a*, with the right-hand ordinate and the topmost abscissa, the curve of Means and Lerman is *illustrated as a logarithmic function; b, with the left-hand ordinate and the* lowest abscissa (open triangles), the hormone stored in extrathyroidal tissue is shown, as estimated by integration of the decay curve against time; *c*, with the left-hand ordinate and the intermediate abscissa (solid circles) are shown actual observations by Lerman and Salter (100) of the requirement of thyroxine-iodine at several levels of hyperthyroidism. These data are supplemented by data (crosses) of Thompson (158) from another case recovering from myxedema.

1, 2, 3 and *5* are graphically presented in figure 2. The daily utilization, *3*, and the calculated content of the organism, *5*, both rise logarithmically with the basal metabolic rate. The dosage (100) required at a steady state of metabolism is shown below, together with comparative

effects of whole thyroid and thyroxine. The desiccated thyroid, by analysis, contained 29 per cent of its iodine in the form of thyroxine.

Response to thyroxine and desiccated thyroid

MEDICATION	IODINE IN SUBSTANCE USED (MGM. PER DAILY DOSE)		LEVEL OF B.M.R.
	Thyroxine-iodine	Total iodine	
None	0	0	−15
Desiccated thyroid	0.17	0.6	0
$C_{15}H_{11}O_4NI_4$	0.5	0.5	+19
Desiccated thyroid	1.4	4.7	+30
$C_{15}H_{11}O_4NI_4$	4.7	4.7	+60

In the hyperthyroid range the actual thyroxine administered is more important than the total iodine in the thyroid substance used. This is not true in clinical myxedema (102) (cf. p. 368).

Genesis of Thyroidal Activity. There is now no doubt that the physiological equivalent of the thyroid hormone may be produced readily outside of the thyroid, as demonstrated by the data of Lerman and Salter (101), already described in section I. Their observations raise the following questions: *1.* Does thyronine (thyroxine minus all four iodine atoms) exist preformed in serum protein as an essential amino-acid, awaiting iodination? *2.* Does the process of iodinating the protein also change molecular configuration so as to produce physiological activity? *3.* Can the thyroidless organism synthesize iodothyronine molecules from other iodinated residues?

The classical, rapid recovery of these individuals confirms Abelin's report (2) of the production of extrathyroidal hormone. Moreover, the fact that serum albumin, when simply iodinated, can completely relieve human myxedema casts doubts upon the prevailing conception that the gland's function is specifically to manufacture a hormone de novo. But lately, the synthesis from diiodotyrosine has been conducted directly in vitro (17). In vivo, Condorelli (25) found that dogs treated with 0.1 gram of diiodotyrosine daily remained healthy despite thyroidectomy. On the other hand, only feeble calorigenic action can be demonstrated when huge doses of diiodotyrosine are administered to human patients with myxedema (158). There seems to be some peculiar function of protein combination, perhaps in orienting hydroxyl groups or in sealing off the amino-acid chains. Indeed, Cohn, Salter and Ferry (23) noted a strange disappearance of tyrosine acidity on titrating iodoglobin.

Recently, Ludwig and Mutzenbecher (126) have reported the isolation of thyroxine itself and of monoiodotyrosine from alkaline digests of iodinated casein and serum protein. Working with serum albumin, the author has obtained only a substance resembling diiodothyronine and a tetraiodo compound resembling deaminated thyroxine, but giving no Kendall-Osterberg reaction. This work is highly interesting, but as yet too recent to be evaluated from a physiological standpoint.

Ordinarily, however, it is clear that the thyroid gland supplies the organism with the form of thyroid hormone which circulates in the blood. What this naturally occurring form may be is still a matter for conjecture, although the following observations on the calorigenic effect of various iodine compounds have definitely limited the possibilities.

CALORIGENIC ACTIVITY OF THYROID HORMONE IN VARIOUS FORMS. There is little doubt that thyroxine (85) qualitatively reproduces the full effect of the thyroid hormone, because in human myxedema substitution therapy with crystalline thyroxine, judiciously administered, affords complete relief indefinitely. It should be emphasized further that the relief of human myxedema is the most reliable test available for thyroidal activity, and that results so obtained by experienced clinical investigators are more convincing than the measurement of oxygen consumption in animals or than metamorphosis experiments with tadpoles (163) and axolotl. This is especially true if quantitative observations lead to generalizations which are to be applied in the clinic.

Harington and Salter (73) isolated levorotatory thyroxine from thyroid protein. When the d- and l-forms made in Harington's laboratory were tested by Salter, Lerman and Means (139) in human myxedema, no difference in activity could be discerned. In tadpole metamorphosis and in rat metabolism, however, Gaddum (61) found l-thyroxine to be thrice as effective as the d-form. This material also came from Harington. Similarly, Foster, Palmer and Leland (59) found in normal guinea pigs that l-thyroxine is twice as active as dl-thyroxine; hence, they infer the d-form to be inactive, but no direct test of the d-form was made to prove it inert. The author believes that the difference in these results is due to biological dynamics, i.e., differences in rates of excretion, destruction and storage in various species receiving various dosages at different levels of thyroid function.

The Natural Hormone. There remain two puzzling facts with regard to thyroid activity which involve not only the true character of the natural hormone but also its pharmacological assay and dosage. The

first fact, pointed out by Kendall, is that certain samples of desiccated thyroid yield no crystalline thyroxine although they are highly potent physiologically. The second fact is that desiccated thyroid or fresh thyroglobulin may produce more calorigenic effect than does the thyroxine contained therein (73).

In view of these facts, both Kendall and Harington have suggested that thyroxine itself is not the true hormone in its natural form. Indeed, Barger (4) suggested that the thyroid contained a more active substance than thyroxine, i.e., a compound which breaks down during isolation or is converted into the less active thyroxine. Kendall and Simonsen (88) also speak of two hormonal forms: *1*, thyroxine, as we know it, and *2*, "active thyroxine." They suppose that the latter substance is the physiologically active molecule and suggest that it may be a hydroxy-thyroxine. Thus, in the tissues compound *1* becomes compound *2* as the first stage of pharmacological metabolism.

Harington (70) suggests that the true hormone contains both thyroxine and diiodotyrosine, in peptide combination, connected by a link or bridge of other amino-acids. Nevertheless, neither thyroxyl-diiodotyrosine nor diiodotyrosylthroxine, synthesized in Harington's laboratory, is as active as thyroxine itself.

Potency of Thyroid Preparations. The discrepancy between the calorigenic activity of thyroxine and whole thyroid deserves special comment, not only because it is important pharmacologically and clinically, but also because it is still the subject of dispute. Thus Foster, Palmer and Leland (59), working with normal guinea pigs, found only the thyroxine fraction to be effective. Likewise, Thompson, Thompson, Taylor, Nadler and Dickie (159) found that desiccated thyroid on the average yielded only 62 per cent as much activity as intravenous thyroxine. They obtained variable results with different samples of desiccated thyroid.

On the other hand, investigators in Boston (121) (102) (139) (119) have treated several scores of cases of human myxedema with thyroglobulin and its various derivatives and find that, in proportion to the iodine it contains, whole thyroid is distinctly more active in these patients than pure thyroxine. Similar results have been reached by Freud and Laquer (60), using a digestion product of thyroglobulin, "thyranon." More recently, Meyer (122) has confirmed these results in rats and studied the effect of thyroidectomy:

> Male rats were thyroidectomized at about 80 days of age and allowed to progress into hypothyroidism for six or eight weeks. Their oxygen consumption was

used as the criterion of the activity of thyroid medication, the dose of which was adjusted to produce an average increase of 30 per cent in metabolic rate. The normal rat proved to be too variable to serve as a reliable test object. Moreover, thyroidectomized animals were found to be about 25 times as sensitive to a given dose of thyroxine as the normal animal, because the former group required only 0.75 gamma of thyroxine to produce the standard rise in metabolism of 30 per cent. The data lead to the following conclusions:

The thyroxine content of a given thyroid preparation does not permit a definite conclusion as to its calorigenic action. The iodine content seems to be a better guide, provided the substance has not been treated chemically, especially not been subjected to hydrolysis.

There is a species difference in the absorption of thyroxine through the intestinal canal of the human individual and the rat, respectively: within the experimental error, dissolved thyroxine is completely absorbed by the intestine of the rat.

Isodynamic doses of various derivatives and preparations of thyroid have different but characteristic effects on the pulse rate of the thyroidectomized rat, ranging from a very slight increase (30 beats) to an increase of 175 beats per minute above the initial value of 180 to 200 (122) (123).

From his results it appears that the response at higher levels of metabolism is minimal and therefore unfavorable to fine discrimination of effectiveness.

These various results indicate that natural combinations of thyroxine in peptide chains are more potent than their inherent thyroxine would be alone. Salter and Pearson (140) demonstrated this fact by breaking thyroglobulin down with pepsin and then resynthesizing a part with pepsin to form an artificial protein or "plastein." The activity of the peptone (containing iodine chiefly in diiodotyrosine form) was increased sevenfold by the reverse synthesis (137). Kendall (86) has suggested that the presence of the peptide chain prevents thyroxine from escaping (to the extent of 75 per cent) from the body, and thus increases its effectiveness. This explanation would relieve one of the embarrassments of explaining activity in diiodotyrosine. Clutton, Harington and Yuill have prepared a synthetic combination of protein with thyroxine (21), but its activity has not yet been assayed.

IV. Physiological iodine excretion. *Urinary Iodine.* Urinary iodine excretion depends upon the iodine intake in food, water and air. Thus, in a district with 0.09 gamma per cent in drinking water, the 24-hour excretion was 47 gamma, whereas in another district with 8.9 gamma per cent in the water it was 185 gamma (133). Consequently, it is more profitable to study excretions in districts where water and flora contain only moderate concentrations of iodine. Under

stable conditions in such districts, individual variation is small—not over 10 to 15 gamma in 24 hours. Normally, about half of the total iodine leaves the body by way of the urine (24). A few representative observations follow.

In Columbus, Ohio, 9-year-old children excrete approximately 41 gamma daily and adults 50 gamma (31). In Lwów, values below 20 gamma are found only in goitrous individuals, and values above 50 gamma frequently accompany thyrotoxicosis (36, p. 114). Nevertheless, 70 gamma occasionally may be found in the urine of normal individuals. In Germany, Isenbruch (82) found 6 gamma per cent iodine concentration in the urine of nursing infants. Goats (27) excrete 21 gamma daily and dogs (151) (153) between 8 and 67 gamma, depending on the animal's size. In cattle enjoying an iodine-rich fodder, however, urinary excretion may reach 600 gamma (141). According to McClendon (116), twice as much iodine may be excreted by day as by night. Indeed, 24-hour urine samples are essential in careful studies of iodine metabolism because of hourly variations (143). No doubt, diurnal assimilation of exogenous iodine is largely responsible for such fluctuations. In fasting animals excretion is more uniform, and declines but slowly.

Most, if not all, of the urinary iodine is present as iodide. No thyroxine can be detected in the urine (50), but whether any diiodotyrosine is present normally is not yet clear.

Fecal Iodine. Because almost no iodine is excreted in the feces of fasting men, it seems clear that fecal iodine is almost entirely of exogenous origin. When large doses of thyroxine are administered intravenously the large bowel actively excretes part of the iodine (147). The bile also contributes to fecal iodine, because the iodine in it may increase from 9.2 gamma per cent, fasting, to 45 gamma per cent after feeding (42) (40). A large part of the biliary iodine is reabsorbed so that there is a "circulation" of iodine analogous to the "circulation" of the bile salts. Of course, unabsorbed iodine in foodstuffs also contributes to the fecal iodine concentration. Consequently, in diarrhea more iodine is eliminated, along with unabsorbed foodstuffs, whereas in constipation relatively little iodine is eliminated. On a routine diet, Scheffer (143) (144) found only from 2 to 10 gamma iodine per 24 hours, namely, 3 to 9 per cent of the total iodine excretion. Cole and Curtis (24) found variable but larger amounts, i.e., from 6 to 27 per cent of the intake.

In animals, fecal iodine appears to be small, as judged from the following values for 24-hour excretion: for the hog, 1.8 gamma (141);

for the goat, 3 gamma (27); for the rabbit, 9 to 12 gamma (36) (171). On the contrary, in cows eating large amounts of iodine-containing fodder, Fellenberg (56) found as much as 1300 gamma per 24 hours.

The iodine excretion of normal individuals is increased by factors, like exercise, which augment total metabolism. After exertion, urinary excretion may reach double the control value (116), provided excessive sweating does not divert iodine elimination to the skin, as often happens. It is possible that the increased blood and urinary iodine is due to thyroid secretion (36) (171). Kommerell (92) found no increase in basal metabolism after exercise in thyroidectomized animals. This phenomenon deserves further study, in view of the prevailing opinion that the thyroid exerts its effect only after a latent period.

Other factors which increase total metabolism also increase iodine elimination. For example, it is said that mental excitement alone may double the excretion (56) (167). Likewise, a fever of 39°C. may treble the excretion (96). Very high iodine excretions—as much as 3000 gamma daily—have been observed after severe operations like thoracoplasties (30) (29), and there is an associated hyperiodemia which begins within a few minutes after operation and lasts for several days. McCullagh (117) believes that this extra iodine comes from the thyroid, whereas Curtis and Phillips believe it to be of extrathyroidal origin. The high iodine elimination of thyroid disease will be discussed in another publication (171).

Excretion by Other Routes. The iodine in milk, in sweat and in saliva has been discussed in Section II. Under normal living conditions near the seacoast, these media play a minor part in the total iodine excretion.

Courrier and Aron (26) believe that the thyroid hormone can be transmitted through the milk of bitches and so affect the histological status of the thyroids of puppies. The possible amount of thyroxine so transmitted has been estimated by chemical analysis and, if present at all, must be exceedingly small (44).

Fasting Iodine Excretion. Fasting has relatively little effect on the total excretion of iodine in a well nourished individual. To be sure, the fecal iodine rapidly disappears; only 0.4 gamma per day is found. The iodine reserves, however, maintain a normal urinary excretion for many days or weeks, depending on the original richness of the diet in iodine. The average "endogenous" urinary iodine excretion was found by Sturm (151) to be 18 gamma in dogs which had fasted for four days. In thyroidectomized dogs, however, fasting produces a rapid, marked

drop, and the total excretion may approach zero. Presumably the greater part of endogenous iodine elimination is linked up with thyroid activity, directly or indirectly. Of course, after a depletion of iodine reserves by fasting, restoration of a normal diet is accompanied by a temporary retention which causes a negative iodine balance.

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THE RÔLE OF POTASSIUM IN PHYSIOLOGICAL PROCESSES

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We are still far from being in a position to present the known facts concerning the physiology of potassium from the point of view of any well developed theory of potassium behavior. Nor is it possible in these few pages to describe or even mention more than a very few of the infinite number of "effects" of K which have been observed in connection with almost all known physiological processes. The various attempts which have been made to explain these effects of K in terms of the fundamental properties of membranes and cells would fill an entire review. An arbitrary selection must therefore be made of a few aspects of the subject which seem to be of particular interest to vertebrate physiology.

As is well known potassium is of the soil and not the sea; it is of the cell but not the sap. Macallum (253) has discussed the various explanations which have been advanced to explain the relative scarcity of potassium in the sea. The reason for the great abundance of potassium in living cells is quite different and still unknown. It is not within the scope of this review to discuss in detail the various theories of potassium accumulation. It will suffice to state here that body cells in general appear to be more or less freely permeable to potassium which presumably stays where it is because no other cation can get in to take its place and because the anions with which it is combined are unable to escape.

The permeability of tissue cells to potassium is shown by the rapid disappearance from the blood stream of injected potassium. A more direct proof is afforded by injection of the artificial radioactive isotope of potassium. In such experiments Joseph, Cohn and Greenberg (205) have shown that soon after injection the marked K reaches a high concentration in the liver after which it slowly moves out of the liver and the concentration in the muscles and in the whole blood slowly increases. A few observations have been reported which show (144) that the marked K is about equally concentrated in liver and muscle

of rabbits after 24 hours but indicate that only about 8 per cent of the muscle K has exchanged and only about 3 per cent of the red cell K. It has been found, in rats, however, (298) that after 10 hours all the muscle K and half of the red cell K has exchanged with the marked atoms. Of greater importance is the fact that after 1 hour a larger fraction of the total K in the liver is radioactive than in the plasma or the carcass as a whole (298). In explanation of this fact it may be postulated that the visceral organs immediately after injection take up or ingest an isotonic solution of potassium of high radioactivity and later give it up slowly by cation exchange to the plasma and thence to the other tissues like the muscles, the red cells and brain. The muscles, therefore, ingest very little excess K but exchange their K fairly rapidly with plasma K. Red cells and brain neither ingest nor exchange K rapidly. All the visceral organs ingest K rapidly but exchange it with the blood about as rapidly as muscles. In any event even the red cells are more or less permeable to K (in rats). Their permeability to Na has recently been shown by similar experiments with radioactive Na (81). Chemical evidence of permeability of red cells to K is not lacking (214) (160), but is still exceptional (308).

Nevertheless, in spite of this demonstrated permeability to K and Na the Donnan membrane theory assuming complete cation impermeability, appears to explain the electrolyte equilibrium in red cells satisfactorily.

The theory of the electrolyte equilibrium across the cells of muscles and other tissues seems to be in a somewhat similar predicament. The most acceptable theory to date has been based on the assumption that the cells are permeable to K but not to Na and are impermeable to anions (281). A recent modification of this theory, which seems to be a considerable improvement, is that described in the preliminary communication of Conway and Boyle (83) who propose that the membrane is permeable to K and the monovalent anions but impermeable to Na and to all other anions.

The most obvious difficulty with this theory is the finding (164) that the large amounts of necessarily intracellular Na found in muscles of rats raised on a low K diet readily exchanges with injected radioactive Na. Here again, in spite of this demonstrated permeability to both K and Na, the theory of Conway and Boyle seems to explain satisfactorily a good many features of the muscle electrolyte balance. Some of these will be referred to later.

The importance of K in the interior of cells. Since all animal cells contain a large amount of K it is pertinent to inquire whether this serves any special function for which Na would not be equally suitable.

In red blood cells it is evident that K plays no essential part which cannot be served equally well by Na, for the corpuscles of cats and dogs contain little more K than the plasma and still function adequately as oxygen carriers. Clark (77) asserts that the frog heart can continue to beat after losing half of its K providing that a trace of K is present in the perfusion fluid. Muscle cells have been observed in K-deficient rats which still contracted even though half the potassium inside the cells had been replaced by Na (165). Such rats, however, are not healthy and it is impossible to say at present to what extent the contractility of the muscles was altered by this change of cation. Moreover it is possible that the potassium in the myofibrils themselves was not lost but only the potassium in the sarcoplasm. The greater solubility of muscle proteins in potassium as compared to sodium salts (162) suggests another possible advantage for a muscle with K as the chief cation. In the liver cells it has been shown that glycogen cannot be deposited unless accompanied by appropriate amounts of K and water (114) but it is probable that Na would serve equally well if it could penetrate the cell. The chief if not the only definite advantage of potassium depends upon its greater mobility, a difference which exists even in aqueous solutions but which is intensified 4.5 times (272) within a collodion membrane. This may make possible the separation of Na and K by the cell membrane. The resulting differences in the concentration of K cause potential differences across the cell membrane and probably account for the properties of excitability and conductivity. The predominant effect of K as compared to other ions upon potential differences in muscles confirms this interpretation. (For references see previous review, 112.) Since no other cation can entirely replace K in the interior of cells without interfering to some extent with the cell function this particular cation must be granted a certain degree of indispensability for life. Even rubidium with a mobility still higher than that of K cannot serve the same purpose.

Isotopes of K. Interest in potassium has been much enhanced in recent years by the discovery of the natural existence of the rare radioactive K^{40} isotope in addition to the heavier K^{41} and the ordinary K^{39} isotopes. This finding in turn led to a recalculation of the half life of this radioactive isotope as 1.67×10^8 years, a much lower value than was previously obtained when all the radioactivity was attributed to the 579 times more abundant K^{41} isotope (44). The K^{40} isotope must disintegrate into Ca^{40} and from the K^{40}/Ca^{40} ratio in the earth's crust the age of the earth has been estimated as 3×10^9 years, a figure in fair agreement with values obtained by other methods (44). Moreover it

appears that the heat of this disintegration in early times must have played an important rôle in maintaining the temperature of the earth. Since all the Ca on the earth came originally from K^{40} it may be said that the biologically important ratio of these two physiological antagonists has been continuously changing since the origin of life.

The possiblity that K might be of importance in cells because of the special radioactive properties of its K^{40} isotope, as originally suggested by Zwaardemaker (410), has been thoroughly disproved. It can be calculated that in heart muscle there is only about 1 β ray produced per gram of muscle per second from the K^{40} isotope. Failure of the heart to beat in a K-free perfusate can hardly be due to lack of such discharges because the K content of the muscle cells themselves is not thereby diminished, nor is the K content of the heart as a whole appreciably changed. In addition Glazko and Greenberg (138) have shown recently that such a heart cannot be made to beat by replacing K by radioactive Na although the β ray activity is thereby increased much above normal. Hamilton and Alles (146) could find no physiological effects from the injection of artificial radioactive isotopes in spite of the great increase in the number of β ray discharges. Further review of older evidence against this theory is no longer profitable.

The related question as to whether the abundance ratios of the K isotopes are the same in living tissues as in the ocean or the earth's crust is still under investigation. Brewer (43) by means of the mass spectrograph has studied the abundance of K^{41} from different sources and reports a slight excess in bone marrow, spleen and kelp and a slight deficiency in rat tumors and auricle (238). These small differences amount to less than 2 per cent and have not yet been confirmed. Loring and Druce (251, 95) prepared KCl from 200 potato plants and found its molecular weight to be 40.04 ± 0.1 indicating an excess of the heavier isotopes. Further confirmation of this finding is desirable, especially since Brewer (45) found relatively less K^{41} in potato vines than in other sources of K. Ernst (103) concluded that biological K had a greater effect on a photographic plate than inorganic K indicating thus an accumulation of the radioactive isotope K^{40} in living tissues. Pohlmann (310, 311) however extracted and purified K from tissues by precipitation as the perchlorate and determined the radioactivity with a Geiger-Müller counter. He concluded that K^{40} was not more than 5 per cent more abundant than in control K samples. Similar data have been obtained (239) for rabbit muscle K, for tumor K (236) and in the writer's laboratory (unpublished) for K from human ashes.

A. and M. Lasnitzki (237) compared the effects of substituting biological K extracted from maize, casein and agar for mineral K in the diets of mice, but their results were contradictory and probably indicated no differences.

Certainly to date the literature contains no evidence that the isotopic composition of biological K is sufficiently different from the normal to interfere with the successful use of the radioactive isotopes as tracers.

The potassium balance of the body. The whole body of an adult rat contains 0.25 per cent potassium (243) (166) (245). The K content of the human body has been variously estimated as 0.11 per cent (252) and 0.35 per cent (338). Assuming an average value of 0.25 per cent it may be estimated that the body of a 70 kgm. man contains about 175 grams of K. Of this amount the blood will contain about 8 grams, the blood plasma 0.3 gram and the total extracellular space about 3 grams.

Most of the ingested K is excreted in the urine but small amounts appear regularly in the feces (308). In two babies (340) 8 to 9 per cent of the ingested K was excreted in the feces the absolute amount increasing in proportion to the intake. According to figures of Wiley and Wiley (383) the feces contain regularly about 0.3 gram of K daily, or about $\frac{1}{6}$ of the daily intake of their subject, but only negligible amounts of Na.

The average intake and excretion of K per day for an adult man averages about 3.4 grams (338, p. 371). During a 6-day fast in an 8 year old girl the excretion of K was reduced to 1.08 gram of K accompanied by 10.3 grams of N (128) whereas in a man after 31 days (25) the daily excreted K and N amounted to only 0.6 and 7 grams respectively. During a fast therefore the N/K ratio in the urine has a value of about 10 which is likewise about equal to the ratio of N to K found in normal muscles (128). This indicates therefore a continuous unavoidable loss of K during fasting which is due to the gradual destruction of cells in the endogenous metabolism of the body. After fracture of a limb a similar loss of nitrogen accompanied by potassium has been observed (86).

Since potassium balance can only be maintained by continual excretion in the urine it is not surprising that there should be a large rise of serum K in experimental nephrectomy (297) (156) (261), but not in milder cases of partial ureteral obstruction (98). In human nephritis high K is seen only occasionally in the most severe cases (293) (303) (313) (386). In general, Ca falls slightly possibly because of the high

phosphate which accompanies the high K (409) (408). Usually, however, no disturbance of K is seen even in severe uremia (10) (289). The symptoms of uremia are not therefore due in general to the toxicity of an excess of potassium (1) as has been sometimes suggested. By histochemical methods Menten (270) has found increased amounts of K, particularly in the convoluted tubules, resulting from diuresis or injury.

Dietary potassium. Lack of K in the diet is detrimental and inhibits growth (305) (274) (275) (276) (243) (166) (9) (147). According to Redina (315) rats grow best if the Na/K ratio is 5, the loss of weight resulting from the inadequate diet being increased with either more or less K. The diet used in this case was, however, deficient in vitamins. On an otherwise adequate diet growth can be completely prevented by lack of K and a relatively enormous excess of K can be tolerated without obvious ill effects. Thus Miller (274) has found no harmful effects in rats from a diet containing 14 times as much K as Na.

In addition to showing inhibition of growth by a lack of K, Heppel and Schmidt (166) have studied the effect of such a deficient diet on pregnancy. While litters were born under such conditions they were promptly eaten by the potassium-depleted mother. Some growth (50–60 per cent of normal) was obtained by substituting Rb for K but such rats became so hyperexcitable that they went into convulsions at the sound of a blast of air and they died prematurely. Except for the K deficiency the diet was adequate for normal growth. Rats raised on a low K diet lose as much as half the K of their muscles, replacing it by Na, but the liver remains unchanged in composition (163).

Likewise man loses K on a salt-poor basal diet supplemented with NaCl (385) and the muscle K of rats on a low K diet is made still lower if NaCl is added to the ration, thus providing in excess a cation which can exchange with K (101). The electrolyte composition of the blood does not change, however, on such low salt diets (345). Numerous investigations have established the general principle that by such a cation exchange intake of K can cause a loss of Na and vice versa (34) (242) (275) (136) (384) (32) (213) (210). Likewise a vegetarian diet which is relatively rich in K requires and is normally accompanied by a higher intake of NaCl (366). The same effect can be nicely demonstrated in the isolated dog kidney (199).

The potassium turnover in the blood. In pancreatic juice the concentration of K is about equal to that in blood plasma, i.e., 4 to 5 m. eq. per liter (203) and after the injection of KCl the increase of K concen-

tration in the pancreatic secretion is about equal to the increase in the plasma (21). In other secretions of the gastrointestinal tract, however, the K concentration is greater than that in the plasma in varying degrees, the values (in m.eq. per liter) being 6.2 to 7.2 in secretions of the jejunum, ileum and colon (24), 6.6 in the hepatic bile (317), 14.0 in the secretion of the esophagus (363) and in the gastric juice (12) and 18.9 in the saliva (weighted average—368). Most of the K secreted in this way into the gastrointestinal tract is reabsorbed lower down so that there is a continual circulation of K and other salts through this route. Using the values given above and the volumes of secretions as estimated by Adolph (3) it may be calculated that about 1.25 gram of K circulates through the glands into the gastrointestinal tract and back again in 24 hours, or all the K in the plasma circulates once every 6 hours. Since, moreover, all the K in the plasma is excreted by the kidney every 2 or $2\frac{1}{2}$ hours in addition to some loss in the sweat, it is evident that the turnover is exceedingly rapid and the constancy of the K level in the plasma becomes all the more remarkable.

Potassium by mouth. Ingested K under certain conditions is absorbed more rapidly than Na (198) presumably because the diffusion gradient into the blood is more favorable. The difference between K and Na in this respect cannot be large because no appreciable difference was found in previous investigations (178) (373) (292). After ingestion K is excreted more rapidly than Na (2) indicating that the K capacity of the body is relatively less "elastic" than the Na capacity. For the same reason potassium salts have proved useful as diuretics (213). Ingested K is also taken up rapidly by the tissues, the increase in concentration of K in the plasma being such as to indicate that the K diffuses into all the body water (39). The ingestion of 12 grams of KCl caused a 61 per cent increase in the plasma 2 hours later (299). Such an increase is not toxic. Similar figures were obtained by Wilkins and Kramer (386). According to Osborne (304), curiously enough, there is no comparable increase of plasma K after the ingestion of 20 grams of KI. With K acetate as little as 2.5 grams per day caused a measurable increase of plasma K (329).

Injected potassium and its toxicity. The efficiency of the body in disposing of excess K is best seen when K is injected into the blood. Such potassium rapidly disappears from the blood (46) the residual increment of K in the plasma being such as to indicate again that the injected K might have diffused into all the body water (392) (381) (115). Wilde (381) has found that some of this K comes back into the

blood again temporarily after 30 to 40 minutes. There is evidence that the liver absorbs more than its proportional share of such injected K (115), but most of the injected K is absorbed by muscles since the rate of disappearance from the blood is said not to be influenced by the removal of the kidney, liver and the alimentary canal (189) (190).

Injected potassium is difficult to trace by tissue analysis. This is due *1*, to the considerable error in the analysis for K, and *2*, to the small amount of K which can be injected. In man the maximum amount considered safe to inject intravenously at one time is only 3 to 4 mgm. per kgm. of body weight (337) (237). This is less than 0.2 per cent of the total 2500 mgm. K present in 1 kgm. of body weight. Perhaps the smallest rapidly injected intravenous dose which has been reported as lethal is 8 mgm. of K per kgm. in guinea pigs (11). For a 2 minute injection 50 mgm. of K per kgm. is a more usual figure (8) (379). The slower the rate of injection the larger the amount of K which can be tolerated. With a very slow infusion of isotonic KCl over a period of an hour the K content of the body can be increased by 220 mgm. per kgm. or about 9 per cent of the normal content (34) (299) before the lethal level of 15 m. eq. per liter of serum is reached (391).

A third reason why injected K is difficult to trace in the tissues is *3*, that it is probably taken into the cells as an isotonic K solution, thus causing little or no change in the concentration of K per kgm. of wet weight. There should be a change on a dry weight basis but changes of dry weight by deposition of glycogen or other means are also possible. Liver analyses before and after injection of KCl have suggested that most of the change in composition was due to an intake by 1 kgm. of liver of 23 cc. of cell water containing isotonic K and a loss of 7 cc. of extracellular water (115).

It is noteworthy that this result is at least qualitatively explained by the Conway and Boyle theory which also predicts that injected K should distribute itself more or less equally in all the body water rather than in proportion to the pre-existent concentration of K as required by the Mond and Netter theory. The liver appears to obey the Conway-Boyle theory better than the muscles in this respect.

If K is injected into the arteries it causes a marked vasoconstriction but is less toxic than when injected intravenously where it reaches the heart in larger concentration and may cause a slowing, auriculo-ventricular block, fibrillation and finally cessation of the beat (263) (145) (167). In passing through the capillaries arterially-injected K becomes mixed with a larger amount of blood before reaching the heart and some

of it is absorbed in the tissues, for venous blood under such circumstances has been found to contain less K than arterial blood.

The toxicity of injected K is diminished by simultaneous injection of Ca (391) or of hypertonic NaCl or glucose (8).

Potassium in growth. There is prevalent an opinion that a high growth rate of tissues is related to a high K content. In mice large rapidly growing tumors had a high K and low Ca content while the reverse was true in slowly growing tumors (80). In general K falls and Ca rises with increasing age of the tumor. In the chicken sarcoma (284) there is in general a fall of K and Mg and a rise of Na, Ca and Cl in passing from the normal through the actively growing periphery of the tumor to the necrotic interior and the same changes occur progressively as the tumor ages. In human tumors the K content has been found uniformly higher than in the corresponding normal tissues, and a low K diet has been recommended for cancerous patients (102). The bone marrow has also been reported high in K during the growth of carcinomas in the body (184). Meyer-Dorkin (271) has found some evidence of local stimulation of growth from daily injections of K in mice, and Kaufman and Laskowski (209) have sought to establish some relation between the decrease in the K content of the eye and the decrease in its growth rate, the eye being an organ the function of which is not appreciably changed by age. During pregnancy the K content of the uterus increases with the growth of that organ (390). This is probably due, however, merely to a decrease in the relative amount of non-muscular tissue. In plants also most K is found when growth is rapid (404). A high K content, however, is presumably the result rather than the cause of the rapid growth and there seems to be no evidence that K has any specific effect upon growth except that it supplies an essential component of the new protoplasm.

Rôle of K in muscle contraction. Many investigations have shown that muscular activity leads to a loss of K in exchange for Na. Pertinent literature has been reviewed previously in this journal (112). More recently the main facts have been confirmed in cats (361), in rats (165) and in frogs (58). The expected increase of K in the blood has also been found in isolated perfused muscles (5) (396), in rats (212), in man after exercise (216) and in cats and frogs (author—unpublished). Heppel (165) has shown that the loss of K in muscular activity persists in rats raised on a K-deficient diet and that the percentage loss is much larger in young than in adult rats.

The significance of the loss of K which apparently accompanies all

activity of skeletal muscle is still somewhat problematical and the information concerning it is still incomplete. The loss of K is in general proportional to the duration and the intensity of the contraction although an incomplete tetanus liberates more K than a complete tetanus, —as if rhythmical activity assists in some way in the liberation (114 a). In a series of twitches the rate of loss of K diminishes with time, but so does the amount of tension developed. Possibly the progressive loss of K is one of the factors which causes the intensity of contraction to decrease. The data do not preclude the possibility that after an hour of stimulation some contractions may continue without further net loss of K. Presumably a steady state is reached in which the loss due to contraction is just equal to the gain due to recovery. Such is probably the condition in the heart. The gain of water on stimulation reaches a maximum after 5 to 10 minutes after which time contraction continues without further gain (actually with a loss) of water (165, cf. also 361). So far as we know, however, the activity of skeletal muscle is always accompanied by a loss of K.

These results make it probable that the loss of K is intimately connected in some way with the contractile process or the immediate recovery processes rather than with the neuromuscular transmission of excitation. The magnitude of the loss, amounting in young rats (165) to 30 per cent of the total muscle K, seems much too large to be accounted for in nerve endings only. Nevertheless Reginster (316) reports experiments showing that in frog muscle stimulation though not sufficient to cause a demonstrable actual loss of K from the muscle, may nevertheless cause a definite increase in the amount of K which can be removed from the frozen and pulverized muscle by a single extraction with water, i.e., an increase in free and a decrease in bound potassium. He further showed that this apparent increase in free K has no relation to the contractile process because it persists even after the complete inhibition of contraction by curare. This surprising result needs confirmation but in any event it does not contradict the conclusion that the actual loss of K from active muscle is dependent upon the contraction of the muscle, for when the contraction is inhibited by curare the loss of K also disappears (Wood, Collins and Moe (396) in perfused dog muscle; Fenn, Koenemann and Sheridan (118) in perfused frog muscle).

A loss of K during stimulation has been explained as the result of an increase in permeability, but this theory has encountered various difficulties (112). According to the Conway-Boyle theory such a loss would occur as a result of an increase in the number of permeable anions.

Loss of K during activity of other excitable tissues. In non-medullated nerve a loss of K on stimulation has been found (85) (401). In medullated cat nerve in vitro it has not been possible to demonstrate such a loss (113). In the sympathetic ganglia, however, (365) there is evidence of diminished K content on the stimulated side. It seems not unlikely therefore that excitation permits a surge of potassium ions through the membrane which then escape into the blood unless diffusion is too much delayed by a medullary sheath.

In *Nitella*, Hill and Osterhout (174) have postulated a similar surge of potassium ions through the membrane in explanation of the action potential. They have also found evidence for the existence in the surface of these large plant cells of some substance, possibly a compound of potassium, the presence of which is essential for the irritability of the cell (173) (175). This compound is removed by treatment with distilled water but can be restored by various means including the treatment of the cell with blood serum (307). Direct experimental evidence for a loss of K on stimulation of these cells has not yet been reported.

In the salivary gland Wills and Fenn (388) found no apparent decrease in K content of glands stimulated through the chorda tympani nerve although a decrease was quite evident after pilocarpine stimulation. In this case the loss of K in the saliva cannot be replenished quickly enough from the blood whereas in electrical stimulation the output equals the intake. The behavior of the glands, therefore, is not contrary to the rule that a loss of K is inevitably associated with activity.

Heart muscle like skeletal muscle probably loses K during activity for low K contents have been found in the over-worked hearts of cardiac patients after death (61) (387) (151).

The possiblity of loss of K from smooth muscle or the central nervous system during activity has not been adequately investigated. Pichler (309) however reported no decrease in K content of the frog brain and cord after strychnine as compared to normal or urethanized frogs. After urethane, however, a larger fraction of the total K could be extracted in 96 per cent alcohol.

The rôle of K in neuro-muscular transmission. An increase of both K (loc. cit.) and acetylcholine (87) has been detected in the venous blood from stimulated muscle. The same is true in the heart after vagal stimulation [for K (194) (247) (38) (131); for acetylcholine (250) (121)] and in the superior cervical ganglion [(365) for K; for acetylcholine (55)]. This finding, however, is probably not peculiar to nerve endings for acetylcholine has also been detected coming from stimulated nerve trunks or their cut ends (26) (29) (27) (248), or present in the nerves

in increased amount as a result of stimulation (288). The increasing evidence of this sort makes it likely that this is a normal physiological process in spite of the contrary view which has been expressed (126). Evidence, such as it is, for a loss of K from stimulated nerve trunks has already been cited. These facts do not suggest therefore that the process of conduction across a synapse or myoneural junction is any more hormonal in nature than the process of nerve conduction itself. Nevertheless the concentrations of both K and acetylcholine appear to have important pharmacological effects in the process of intercellular nervous transmission, and it may well be that under different conditions any one of the 3 factors, K, acetylcholine or electrical change, might become by itself the decisive factor in permitting or preventing the passage of excitation. Space does not permit discussion of this question here except as it relates to K.

Potassium possesses certain properties resembling those of acetylcholine which fit it to act as a neurohumor. Brown and MacIntosh (56) have shown that application of K to nerve fibers can cause excitation and Cicardo (73) has produced a contraction of the gastrocnemius muscle by injection of KCl into the sciatic nerve. Buchthal and Lindhard (57) in the isolated muscle fiber of the lizard have found that the application of KCl to the end plate causes a rapid tetanus-like contraction while on the muscle fiber itself there is no effect except for the contracture produced by higher concentrations of K. Brown (54) has observed the twitch-like contraction of cat muscle caused by close arterial injections of KCl followed by an electrically "silent" contracture. Moreover, Szent-György, Bacq and Goffart (354) have reported an experiment in which the hind legs of two frogs were perfused in series; contraction of the muscles of the first frog was followed after a suitable interval by irregular contractions of the muscles of the second frog. The result was obtained only when veratrine was present which sensitizes muscle to K (17) and the experiment succeeded in the absence of eserine so that any acetylcholine would have been destroyed. The authors conclude that K is the transmitting agent in this case.

The writer has sometimes found the concentration of K in the blood from stimulated frog legs higher than that in the arterial blood by 100 per cent and in the presence of veratrine this increase in concentration might well be sufficient to cause stimulation. In the experiment of Szent-György et al. (354), therefore, K probably did act as the agent transmitting excitation, but this is no proof that K has this function normally. The fact that curare abolishes nerve muscle transmission

but does not abolish the stimulating effect of K (73) (54) seems to rule out this possiblity. Brown and Feldberg (55) have advanced the same argument against the contention of Eccles (97) that K is the transmitting agent in the superior cervical ganglion.

However, even though K cannot properly be regarded as a humoral agent for neuromuscular transmission it undoubtedly plays an important rôle in the neuromuscular junction or synapse. Thus the injection of KCl (389) (110) may serve to reëstablish contractions from nerve stimulation in a muscle previously paralyzed by curare. The striking effects of K in curing or preventing an attack of familial periodic paralysis (280) (171) (7) are to be interpreted in this sense. During spontaneous attacks of this disease the K in the blood regularly decreases (129) (4) (130). The attacks may be induced by procedures tending to lower blood K such as injection of adrenalin, insulin, or of sugar (339) (400) or a high carbohydrate meal. Injection of $CaCl_2$ aggravates the attacks, whereas injection of K or muscular exercise (which liberates K) tends to prevent them. (Patients testify that when they feel an attack coming on they can sometimes "walk it off" (280).)

Some favorable effects of K have also been observed in myasthenia gravis (372) where the dramatic effect of prostigmin indicates clearly that the difficulty is myoneural in its location. This effect may be due to a liberation of acetylcholine by the K or to the ability of K to inhibit cholinesterase (269). However Minot (278) has found guanidine also effective in relieving myasthenia gravis although it has no inhibitory effect upon cholinesterase. Both guanidine and potassium (and acetylcholine) contractions (132) of muscle are inhibited by Ca, and guanidine and potassium are synergistic in their effect on muscle (125) (399) (320). It is therefore possible that guanidine resembles K in its ability to liberate acetylcholine and owes its effectiveness in myasthenia gravis to this fact.

Feldberg and Vartiainen (123) have suggested that the persistent excitatory condition in a ganglion following the transmission of an impulse may be due to K. In skeletal muscle Feng et al. (110) have shown that the potentiation of the twitch by a preceding tetanus (post-tetanic enhancement) can be duplicated by the application of K.

Since both K and acetylcholine are formed at nerve endings it is possible that both are formed simultaneously or that one causes the liberation of the other. It is unlikely, however, that acetylcholine could liberate K even though it acts like a base and could exchange

places with K in organic complexes (323). In skeletal muscle certainly the amounts of K are several thousand times as large (in equivalents) as the amounts of acetylcholine liberated so that cation exchange could not account for more than a minute fraction of the total K which escapes during stimulation. Houssay, Marenzi and Gerschman (191) (192) have reported an increase of K in the blood after injection of acetylcholine, but even if true this might be attributed to the muscular twitching which results from the injection.

On the other hand, a liberation of acetylcholine by injection of KCl has been well established in the superior cervical ganglion, the salivary gland, and the tongue (55) (120), in the heart (28) and in the placenta (68). In the placenta it was shown that the effect was due to a transfer of acetylcholine from tissue to blood rather than to a new production of acetylcholine.

If however, potassium represents the normal mechanism for the liberation of acetylcholine then the potassium involved in this process must be scarcely detectable in amount for in skeletal muscle curare abolishes all perceptible loss of K during stimulation (396) (118) without abolishing the appearance of acetylcholine (50) while in the heart during vagal stimulation atropine has the same effect (241). Thus in both heart and skeletal muscle the liberation of K is *largely* the result rather than the cause of the acetylcholine effect. In the skeletal muscle this K is concerned with contraction while in the heart it is apparently concerned with the process of inhibition.

While the excitation of the vagus nerve certainly liberates K in the heart (as has already been mentioned), Wollheim (395) has found that it causes a fall of K and a rise of Ca in the blood of the portal vein after sympathectomy. Opposite effects on K and Ca were produced by sympathetic stimulation after cutting the vagus. The meaning of these facts is not yet clear.

In the salivary gland an increase of K is known to cause a liberation of acetylcholine (120). Brock, Druckrey and Herkin (51) in studying the stimulation of gland slices by acetylcholine or adrenalin (as indicated in respirometers by the resulting increase in O_2 consumption) have shown that only a single stimulation is possible in a K-free medium. Subsequent addition of K permits a repetition of the previous stimulation. This does not prove, however, that K is an essential specific link in the excitation chain for either acetylcholine or adrenalin since any other injurious but reversible change in the solution which rendered the gland non-excitable could have a similar effect. In a later paper, in fact, the

same authors (52) found that a sugar-free medium acted in the same way. It is a striking fact, however, that the glands do not lose their excitability even after prolonged soaking in a K-free medium until after they have been stimulated once.

According to an attractive theory of Zondek (405) (406) the parasympathetic and the sympathetic nervous systems act by changing the K/Ca ratio in the tissues. A relative increase of K is supposed to result from parasympathetic stimulation and to cause its effects and vice versa. While many facts can be reconciled nicely with this theory an equally large number of facts appear to be at variance with it (16) (200) (18). There may be some truth in this theory under special conditions but evidently it is not a universal law, and further difficulties with it will appear in what follows.

Potassium and adrenalin. One of the difficulties with the Zondek theory is the fact that the injection of K (which ought to have only parasympathetic effects) liberates both acetylcholine and adrenalin from the adrenal gland. Presumably the acetylcholine serves to stimulate the gland and to liberate the adrenalin (122). That adrenalin does appear after injection (especially arterial injection) of K and is responsible largely for the resulting pressor effects has been shown in many investigations (230) (158) (18) (195) (208) (169). Conversely the injection of adrenalin, by its effect on the liver, liberates potassium (260) (189) (190) (92) (93) (94) (47). According to Hug (195) the effect of KCl in liberating adrenalin is antagonized by $CaCl_2$ and Kusnetzow (230) perfusing isolated cattle adrenals found a similar inhibition by $CaCl_2$ although Ba and Sr had stimulating effects even more marked than K. Hermann et al. (169) find that NH_4 and Rb and to a lesser extent Li and Cs (but not Na) act like K in this respect. Bacq and Rosenblueth (18) and Katz and Katz (208) find evidence that $CaCl_2$ like KCl liberates adrenaline. This may explain the synergism between Ca and adrenaline which has frequently been reported (264, p. 287).

The effect of K in liberating adrenalin is in part responsible for the rise of blood pressure from arterial injections of KCl which is usually absent from intravenous injections. The pressor effect of intra-arterial KCl is increased by previous injection of adrenalin (266) and vice versa (377). Kylin (231) however, in diabetic patients finds a smaller response to adrenalin after KCl.

Camp and Higgins (62) (63) have demonstrated a widespread parallelism between potassium and adrenalin in their effects upon the blood pressure, heart, etc. They attribute to adrenalin the function of regu-

lating the distribution of K in the body and show that K exhibits its characteristic effects even in the absence of the adrenals. These facts are contrary to the theory of Zondek. In the contraction of the iris, however, the effects of adrenalin and K have been shown to be antagonistic (336). Furthermore the rise of blood pressure resulting from adrenaline is not due to the K which it liberates but may occur equally well with a falling level of blood K (46).

Injected K may therefore cause a rise of blood pressure by a direct peripheral effect or by liberating adrenalin. It may do so also, if the concentration is sufficient, by a central effect (170) (364), and this pressor effect may be duplicated by an injection of K into the floor of the fourth ventricle.

On injection of adrenalin the K first rises and then falls in the blood. The initial rise is so transitory that it can only be detected in man during the first 30–90 seconds after injection, (47) and the rise is greater in arterial then in venous blood because some of the K is removed in passing through the tissues. Because of the transitory nature of this rise, Keys (217) concluded that man differs from the laboratory animals in his response to adrenalin. In spite of this initial rise of K after adrenalin, the subsequent prolonged fall is often a more striking feature of the injection (402) and its cause is unknown unless it represents an over-compensation on the part of the muscles which remove the excess K from the blood (192) (47).

The function of this mobilization of K by adrenalin may possibly be related to muscular exercise which is known to cause the secretion of adrenalin (64). It might be supposed therefore that adrenalin assists in recovery from exercise by returning K from the liver to the muscle. If that is so then adrenalin should cause an increase in the K content of the skeletal muscle. The only direct experimental evidence on this point is apparently the paper of Sugimoto (353) (available to the reviewer in abstract only) who reported an increase in the K content of the quadriceps muscle after adrenalin. In addition adrenalin has been found to inhibit the loss of K and PO_4 from frog muscles immersed in Ringer's solution (and to decrease the electronegativity caused by the application of K to muscle) while parasympathetic drugs had the opposite effect (302). In the small intestine of dogs adrenalin decreases while pilocarpine increases the amounts of K and Mg which can be extracted by trichloracetic acid (104). Finally Dresel and Wollheim (91) in the isolated guinea-pig intestine have reported that adrenalin decreases the K content of the bath while increasing the K content of

the muscle strip, the changes being, however, rather small and irregular. Although these experiments with smooth muscles are not quite pertinent to the question the evidence as a whole may be regarded as possibly suggestive of some rôle played by adrenalin in restoring K to fatigued muscle.

Potassium and asphyxia. Closely related to the adrenalin effect is the effect of asphyxia upon K. Generalized asphyxia of an animal causes a stimulation of the adrenals and a liberation of K (191) (192) (411) (66) (285) (90). Houssay, Marenzi and Gerschman (192) have shown further that the rise of K is dependent chiefly upon the presence of the liver. Presumably therefore adrenalin participates in this reaction.

It is not, however, certain that all the potassium comes from the liver since a loss due to asphyxia has also been observed in the heart (89) (78) and in cat skeletal muscle (20) (119). It appears likely therefore that all tissues tend to give up some K during asphyxia, the greatest release being, however, from the liver as a result of a special adrenalin mechanism. In muscle, however, only a small loss of K was found during circulatory slowing and this loss did not appear to increase with more prolonged stasis (119).

Perhaps the most striking feature of these facts is that the release of K is so small in spite of the large concentration gradient existing between cells and plasma and the large mass of cells surrounding the blood stream. Evidently K is not retained in the cells altogether at the expense of oxidative energy. This is supported by a number of other observations. Frog nerve may be immersed in Ringer's solution in the absence of O_2 for 5 hours without losing more K than is lost under similar conditions in O_2 (111). Likewise in crab nerves Cowan (85) has found no loss of K during temporary asphyxia although prolonged anoxia does have this effect. In frog muscle perfused with an O_2-free solution the loss of K due to anoxia was too small to detect in the perfusate (223). In the frog heart a small initial loss of K was observed in asphyxia (78), but this diminished with time and in any event the loss amounted to less than 3 per cent of the total K present. Red cells retain their K in spite of treatment with oxidative poisons (88). In the plant *Halicystis*, Blinks, Darsie and Snow (31) have shown changes in the membrane potential due to anoxia but have concluded that lack of O_2 decreases the high mobility of K (relative to Na) in the surface, increases the electrical resistance of the protoplasm and protects the cell from loss of its contained electrolytes. Possibly the small

demonstrated loss of K from muscle or other cells in asphyxia is to be regarded as only a secondary effect—a new equilibrium resulting perhaps from an increased concentration of lactic acid or other substances inside the cell. An eventual loss of K after a period of anoxia sufficiently prolonged to kill the cell can hardly be regarded as evidence of a specific connection between oxidation and retention of K.

The high potassium content (29.8 mgm. per cent) of human blood drawn from the heart immediately after death is probably to be regarded as the result of a terminal asphyxia (333). It is possible also that the loss of K from the heart observed by Kehar and Hooker (211) during ventricular fibrillation is likewise the result of diminished coronary flow. If so it is consistent with other observations on asphyxia in that the loss of K was not progressive but tended to disappear as fibrillation continued.

Hemorrhage and shock. As blood is progressively withdrawn from an animal interstitial fluids and later cell fluids move into the vessels to maintain the blood volume and the plasma K concentration increases. For small blood losses the rise of K in the blood is absent or negligible (although there is a K diuresis (352) unaccompanied by a corresponding loss of nitrogen). Kerr (214) found no change of plasma K but a large increase in the K content of the cells (indicating permeability to K). With larger blood losses the plasma K rises more and more steeply as the loss of blood increases (20) (92) (357) (412) (413) (191) (192). Since the increment of lactic acid in the blood is greater (in equivalents) than the increment in K (108) it might be supposed that K came into the blood to neutralize the lactic acid. In spite of this added base, however, Johnston and Wilson (204) have found that the decrease in alkali reserve is greater than can be accounted for by the increment in lactic acid.

Houssay, Marenzi and Gerschman (191) (192) have shown that the K increases in hemorrhage only when the liver is intact. This suggests that the rise of K may be due to the action of adrenalin which is known to be secreted in hemorrhage (53) as it is in asphyxia. The situation in hemorrhage, however, is also similar to that of adrenalectomized animals which lose NaCl and H_2O and must make up the deficiency at the expense of intracellular fluids containing K. For the same reason a rise of plasma K is observed after intestinal fistulas or high intestinal obstruction (30) (334) (335) where the salts contained in the intestinal secretions are not reabsorbed and are therefore continuously lost to the body. Under such conditions the administration of NaCl is bene-

ficial but KCl is toxic (127). In general, K is high also in traumatic shock (413) at least if sufficiently profound (30), in anaphylactic shock (394) (330), in hyperthermic shock (30) and in histamine shock (330) (229) (357). Another similar condition where a rise of K might be expected is heat cramps (355) but according to the data so far available a high K does not appear to be an invariable accompaniment of this malady. An intraperitoneal injection of glucose solution likewise depletes the blood of NaCl and leads generally to a rise of K in the plasma (322). After a rise of K due to hemorrhage an injection of salt solution or of blood causes a return of K to normal (357), and Scudder and Zwemer (334) found the same result with NaCl after an intestinal fistula. This finding supports the theory that the rise of K in hemorrhage represents an attempt to build up the blood volume at the expense of cell fluids after extracellular fluids are exhausted.

The adrenal cortex. Numerous investigations have clearly established the fact that removal of the adrenal cortex leads eventually to low Na and high K concentrations in the serum due to excessive excretion of Na and Cl and defective excretion of K (22) (157) (154) (249) (295) (296) (258). In many respects the symptoms of adrenal insufficiency resemble those of toxic doses of K (414) (415). Potassium is abnormally toxic after adrenalectomy (6) (382), and a low K diet as also a high Na diet is favorable to survival (154) (79). Injection of cortin lowers serum K by decreasing the excretion of Na and increasing the excretion of K (155) (360) (359).

From the point of view of the physiology of potassium it is of prime importance to determine whether the changes in the concentration of this ion which have been described for adrenalectomized animals are directly determined by the presence or absence of cortin or whether cortin acts only on the excretion of Na and Cl, all the other changes being secondary to the resulting decrease of blood volume. A good case can be made out for the latter view for a loss of Na and Cl leads to a loss of water thus simulating the conditions of hemorrhage and shock.

Other observations indicate however that some definite effect of cortin upon potassium both in the kidney and in the tissues has to be postulated in addition. Thus even after removal of the kidneys from adrenalectomized rats the administration of cortin caused the usual fall in the concentration of K in the serum (196). Direct analysis of tissues of adrenalectomized animals for K has provided further evidence. Some investigators have found no change in the potassium content of the muscles in the absence of the adrenal cortex (414) (258), but this may

have been due to an increase in water content of the muscles. Allowing for this factor an increase of K concentration in the intracellular muscle water has been found (160) (150) (287). There is also an increase of K and decrease of Na in the red cells (160), a decrease of K in the liver water (150) and a decrease of K in the liver and the heart (258). Administration of cortin reduces the high muscle potassium to normal (150). A high muscle K might be due to a direct effect upon the muscle potassium equilibrium or to accumulation of excess potassium in the body through failure of the kidney to excrete K, but it could hardly be due merely to failure to excrete Na. There is some evidence therefore that cortin moves K from the muscles to the viscera.

Still further evidence that the adrenal cortex has some specific potassium effect was provided more recently by experiments with desoxycorticosterone (228). After daily administration to dogs for 10 to 20 days the serum K fell to about half the normal value and symptoms resembling those of familial periodic paralysis developed. The paralysis was cured by administration of KCl or withdrawal of the hormone. The relation of K to familial periodic paralysis in human subjects has already been discussed.

Potassium in carbohydrate metabolism. In Addison's disease the blood sugar is usually low and the serum K is high. In other conditions however the sugar and potassium rise and fall together. Thus it has been demonstrated by many investigations that the fall of sugar resulting from insulin is accompanied by a simultaneous fall of K and PO_4 (152) (153) (148) (49) (380) (215) (84) (99) (312) (218). This suggests a formation of hexosephosphate in the muscle and actually the number of equivalents of glucose, K and PO_4 which disappear from the blood are of the same order of magnitude (assuming no simultaneous entrance into the blood). The injection of glucose into the blood of cats and rats lowers K as if sugar were being deposited in tissues in combination with K (124).

This relationship between K and glucose justifies the expectation that K may be high in diabetes untreated with insulin. Unfortunately many reports in the literature leave some doubt concerning the type of treatment employed. It seems probable however that a high serum K is not usually characteristic of this disease (10) (308, p. 787) (318) although some high cases have been reported (314) (348) and both Brems (42) and Kylin (quoted by Brems) have found average values for diabetes slightly higher than normal.

After phlorhizin both sugar and K are low in the blood (206) (403).

Likewise as already mentioned adrenalin liberates both K and sugar from the liver. Snyder and Johnson (346) reported that vagal stimulation tended to cause a decrease in the output of both K and sugar from the perfused liver of the turtle. In many cases, therefore, both K and sugar appear to move into or out of the blood together. One possible reason for this behavior may be that the deposition of glycogen in the liver has been shown to be necessarily accompanied by the deposition of water and potassium (114). The quantity of K so deposited, however, is far less than the quantity of glycogen (measured in equivalents) whereas the blood changes would suggest equivalent amounts. A better explanation is offered by the theory of Conway and Boyle (83) according to which any increase in the indiffusible anions in the cells relative to the diffusible ones would necessitate an intake of both potassium and of water. If therefore inorganic phosphate were combined as hexosephosphate an intake of K would be predicted.

Since carbohydrate metabolism is also under the influence of the pituitary and hypothalamus, some parallel effects upon K might be expected. The data are not, however, unequivocal on this point. Marenzi and Gerschman (259) found a fall of K after hypophysectomy but no increase in K with injections of anterior lobe extracts, where an increase of sugar is expected. Moreover van Bogaert and van Meel (36) reported a decrease of blood K on stimulation of the hypothalamus of dogs after vagotomy.

Carbohydrate metabolism is clearly influenced by the administration of K although some conflicting facts have been reported. The injection of K into cats and rats causes hyperglycemia and lowers muscle and liver glycogen (341) (342) (343). Similar evidence that high K favors glycogenolysis is seen in the observations of McQuarrie et al. (267) (268) that a high K diet in diabetes increases glycosuria and the excretion of water and decreases the sugar tolerance and the blood pressure while Na has an opposite effect. These facts are in agreement with the effect of K in stimulating the liberation of adrenalin which in turn mobilizes both glucose and K from the liver. In the light of these facts it is difficult to interpret the convincing report of Odashima (301) who found that injection of K *lowers* the blood sugar in rabbits and raises lactic acid. A similar fall of blood sugar after intravenous injection of KCl in man was found by Semler (337) and by Kylin and Engel (232). A hypoglycemia after KCl as well as a hyperglycemia after $CaCl_2$ has also been reported (183) (407).

Just as K and glucose rise and fall together under certain conditions

apparently concerned with movement of glucose into or out of the liver so also there are conditions, related chiefly to muscular activity, under which K and lactic acid appear to move together. Odashima (301) found that K and lactic acid are increased by strychnine and decreased by curare. As already mentioned both substances appear in the venous blood from contracting muscles. An increase in the lactic acid concentration in the blood has been reported in histamine shock (100) along with the increase of K already mentioned. In the salivary gland there is indirect evidence to suggest that K is lost from tissues only when lactic acid is simultaneously escaping (52). In asphyxia and after hemorrhage both K and lactic acid increase in the blood and the equivalent concentration of the lactic acid is more than equal to that of the K. It is tempting to suggest that K leaves the liver or enters muscle in company with glucose while it leaves the muscle or enters the liver along with lactic acid. One difficulty with this hypothesis is that after stimulation K leaves the muscle before lactic acid does (118). Other exceptions to such a general rule must, of course, be anticipated. Some support for such a tentative hypothesis is found in the observation (115) that after muscular activity there is a consistent increase in the K content of the liver. The existence of a K cycle from muscle to liver and other viscera, and back again comparable to the Cori carbohydrate cycle was suggested.

Potassium and nerve function. Like most cells nerves contain K in relatively high concentration inside (117) (331). The effects of various salts on the electrical potential measurements indicate that K has a high mobility in nerves (294) (172). Treatment of nerves with K salts produces characteristic changes in their microscopical appearance, the axis cylinders being broadened and swollen whereas in Ca they are shrunken (179) (254) (105). Similar effects in high concentrations of K and Ca have been reported in muscle (207). Again therefore an increase in the concentration of K outside the cells causes an intake of K and H_2O, i.e., cells swell in KCl.

With sufficient excess of K the oxygen consumption of nerve is depressed (69). The threshold of electrical stimulation is in general first lowered and then raised by K (142) (180) (279) (140) (33) (325). Larger amounts of K, of course, cause inexcitability and in general Ca has an effect opposite to that of K. This modification of excitability by K is to be related to its effect upon the resting membrane potential of the nerve which becomes progressively smaller or increasingly negative outside. Thus the effect of K resembles a cathodal polarization (398)

(140) (96) (233) while the opposite effect is produced by Ca and anodal polarization. According to Lehmann (240) K and alkalinity (or lack of Ca) decrease the negative after potential, increase the positive after potential, lower the threshold and cause spontaneous discharge. Alkalinity acts presumably by decreasing the concentration of Ca ions.

In reflexes or central nervous system transmission K has a similar effect in causing stimulation or increasing excitability. With larger doses there is a depression and the effect of Ca is regularly antagonistic to that of K (186) (187) (161) (351) (133) (364) (37) (319) (169) (286) (106). It has also been found that a small amount of K increases the formation of acetylcholine by brain slices while larger amounts inhibit it (257), thus suggesting a different mechanism in explanation of the K effect.

Elsewhere in the body K also shows excitatory effects. The electrical excitability of human muscle has been found to be increased after ingestion of K salts or alkaline Na salts but not after NaCl or NaH_2PO_4 (300). Intraärterial (282) (283) as well as intravenous (337) injections of potassium are effective in exciting pain receptors in the vessel walls. It has been shown that intradermal injections of K phosphate cause more persistent pain than similar injections of Na phosphate (142 a). It is suggested that pain experienced in inflammation is due to loss of K from neighboring cells. The K content of fluids in such inflammatory areas appears to be large enough to justify this hypothesis. Likewise Brandi (40) has found that local anesthetics or lumbar anesthetics may cause pain or headache if injected with too much K. However, attempts which have been made (221) (222) to demonstrate a high cutaneous irritability when serum K is high do not appear altogether convincing. On the other hand, an excess of K diffusing from injured cutaneous cells has been suggested (109) (177) (326) as cause of the observed adaptation of sensory receptors following trauma to the skin. In accordance with this suggestion Rusk and Kenamore (327) have claimed some success in treating chronic urticaria with a high K diet.

Because of the excitatory effectiveness of K on pain endings and the readiness with which muscles lose K during injury or activity it has been suspected that the muscle pain arising in muscle fatigue, cramps or ischemia might be due to an accumulation of K. Maison (256) however has presented some suggestive evidence against this hypothesis by finding that just as much intramuscularly injected K is required to cause pain after fatigue or ischemia as in a normal muscle. It is possible, of course, that K does not accumulate at a constant rate as

fatigue progresses but appears suddenly at a critical stage. If so, the evidence presented was not decisive.

Potassium and muscle function. Numerous investigators have observed an increase in response, a stimulation, or an increase in excitability of skeletal muscle after administration of small amounts of potassium (59) (227) (277) (19) (159). Careful distinction has not always been made between effects on contractility and effects on excitability but Lapicque and Nattan-Larrier (234) have established a decrease in chronaxie and Chao (71) a decrease in rheobase. Furthermore, in measuring excitability, distinction has not always been drawn between effects on nerve stimulation (which usually causes the response when electrodes are laid on muscles) and the stimulation of muscle proper. Carleton, Blair and Latchford (65), however, have measured the strength-duration curve of the alpha excitability of frog muscle and have found that the administration of K causes an initial decrease of rheobase and increase of the time constant, k, of excitability (or a decrease of chronaxie). Subsequently both quantities change in the reverse direction and the muscle becomes inexcitable.

When applied to the nerve or the end plate, K causes an all-or-none response of the muscle. When applied to the muscle fiber itself in larger concentration, it causes the familiar K contracture (57) or may cause the fiber to give graded responses instead of all-or-none responses to electrical stimuli (349). Similar graded responses with K treatment have been reported in the heart (416). The K contracture is accompanied by phosphocreatine breakdown, lactic acid formation and increased O_2 consumption (159) (290), increased heat production (347) and eventually by inexcitability. According to Solandt (347) the heat production is increased by concentrations of K too small to cause a contracture but Hegnauer, Fenn and Cobb (159) believed the thresholds for the two effects were identical. Later (347) a correlation between contracture and the first phase of heat production was found.

The effects of K on smooth muscle cannot be summarized briefly nor can any such summary be really adequate which does not include at the same time the effects of all the other ions and physico-chemical conditions. The complications of the subject are well illustrated by the recent study of Singh (344) which cannot be briefly summarized. An effect observed after increasing K may often equally well be described as due to relatively too little Ca and vice versa. Since there is an optimum concentration for each ion for a given set of conditions, it is frequently possible to obtain contradictory results over different con-

centration ranges. Thus starting at certain K concentrations a contraction may be produced in a wide variety of smooth muscles by both an increase and a decrease of K (356) (200) (378) (201) (76). Since K may have different effects on frequency, amplitude, tone, etc. an intuitive description of the effects on "activity" is difficult to interpret (225) (371). In general an increase in K/Ca appears to increase tone and decrease frequency (405) (185) (182) (255) (132) (23) (344) or increases the response to pituitrin (362). None of the effects in smooth muscle appear to be fundamentally inconsistent with the initial or low-concentration increase and the subsequent or high-concentration decrease of excitability seen in striated muscle. The increase in tone of smooth muscle is probably comparable to the K contracture of striated muscle.

In heart muscle there are similar difficulties but again, acting for a short time or in small doses, K causes an increase in excitability or decrease of chronaxie (185) (219) (82) and may thus increase the heart rate in the frog (75), the oyster (374) and *Limulus* (70). Even in mammals there is some evidence of a slight initial acceleration of rate (41) (63). In dogs this acceleration has been explained as due to extra-systoles (167) which is not inconsistent with an increase in excitability. The usual decrease in rate observed with K (328) (35) may be due to a central increase in vagal tone (167) or to decrease in excitability (increase in chronaxie) (74). In molluscan hearts in K solutions an increase in tone is observed similar to that in smooth muscle (also in crayfish) (246) and with sufficient K such hearts stop in systole (185) (374). In vertebrate hearts, however, the decrease in excitability with high K solutions has a predominant effect and leads to slowing of the beat, heart block, fibrillation and eventually arrest in distole (262) (193) (379) (391) (291) (358) (149) (295).

When a heart has nearly stopped beating due to lack of K and the K-free solution is then replaced by normal Ringer's solution, the heart at once stops beating completely for a time before it resumes rhythmical contractions. This is the "potassium paradox" of Libbrecht (244) which has been repeatedly confirmed on hearts of various animals (60) (410) (220) (378) (70) (393). The heart having "tried" to compensate for lack of K finds itself overcompensated when a normal K concentration is supplied and has to stop temporarily from an excess K effect until readaptation is complete.

The production of ventricular fibrillation by K may be the result of increased excitability or of local increases in the refractory period.

Excess of K stops this fibrillation (379). This paralyzing property of large doses of K has been utilized in the treatment recommended for the ventricular fibrillation resulting from electric shock (188) (379).

As already mentioned K is low in hearts in cardiac failure. According to Hagen (143) therapeutic doses of digilanid C increase the potassium content of hearts. Toxic doses, however, cause the heart to lose K (375) (143) (397) (67). Cattell and Goodell (67) found a similar mobilization of K from skeletal muscle under the influence of ouabain. According to these results it might be supposed that digitalis and K have somewhat similar effects, and Camp and Higgins (62) have indeed shown that K (as well as epinephrine) potentiates the action of digitalis. Others, however, (406) have postulated a close resemblance between digitalis and Ca.

Potassium in blood. There is some evidence that a larger fraction of the plasma K is indiffusible than is the case with Na. This conclusion has resulted from analyses of plasma and its dialysate for Na and K (141) (324) (197). Quite different evidence of a special association of K with some organic anion was obtained by Waelsch and Kittel (370) who found K migrating to the anode in a special cataphoresis apparatus.

An increase of alkalinity has been found to result from the injection of KCl into the blood (377) or into an isolated heart on a Straub cannula (226). $CaCl_2$ under similar conditions causes an increase in acidity. Of course, changes of this sort in venous blood are very difficult to interpret chiefly because of possible changes in blood flow and respiration. According to Glatzel and Mecke (137), however, intravenous injection of KCl in man increases the pH and lowers the alveolar CO_2 tension and the CO_2 capacity at the arterial point. Probably the extra K is taken up by the cells more readily than the anion leaving an excess of acid in the blood and lowering the alkaline reserve. At the same time the respiration is increased more than enough to compensate for the increased acidity, thus explaining the low alveolar CO_2 and the high pH.

In this connection it is worth noting that K and OH^- usually act synergistically in physiological reactions (48) (240) (132). An increase in OH^- concentration may act by lowering the concentration of diffusible Ca or it might favor the penetration of K as KOH. Komiyama (224) however concludes that in their effects upon the heart K and H^+ act alike, both stopping the heart in diastole, while Ca and OH^- both lead to cessation of beat in systole.

Other potassium changes in blood. Under this heading may be mentioned a rise of arterial K on stimulating the central end of the sciatic nerve (13) (15) which persists after denervation of the liver and after removal of the adrenals, thyroid and pancreas. The writer has several times succeeded in confirming the observation on intact anesthetized cats. The mechanism of the effect is not clear.

A fall of serum K has been seen by many investigators after anesthetics (14) (135) (321) (235) and a rise of K after 2-4 dinitrophenol (235). This has been taken to indicate that the K level in the blood follows the metabolic rate as it often does. It may be doubted, however, whether any one such factor can explain all the vagaries of the K ion.

It is perhaps significant that in many cases an increase in K in the blood plasma is accompanied by a decrease of Ca (390) (408) (386) (293) (156) (107). Odashima (301) has also reported that injection of K salts lowers blood Ca and vice versa. This suggests that both K and Ca are regulated in the blood by the same mechanism. In other conditions, of course, as in various kinds of shock where Na is low Ca may be somewhat elevated as well as K (330) (229). An increase of both K and Ca after ingestion of potassium acetate has also been reported (329).

During epileptic convulsions the plasma K is increased and the Mg is low (176). This especially anti-narcotic or excitatory composition of the blood might be the cause of the seizures but it might also be interpreted as the result of the muscular contractions. McQuarrie (265) however has found an increased excretion of K in the urine just before the convulsions begin.

High plasma K values have also been reported in allergy (394), hypertension and bronchial asthma (42), dermatitis (367) and severe infections and burns (350) and after the injection of NH_4Cl (139) (author unpublished). In the case of NH_4Cl the effect is most reasonably explained as an exchange of K for NH_4^+.

SUMMARY

Potassium is an essential part of the physico-chemical structure of the cell. It is not fixed in position but can move about in the body rather freely according to the demands of shifting membrane equilibria. According to the best evidence at present this is a static equilibrium relatively uninfluenced by lack of oxygen. It probably moves in or out of cells when this equilibrium is disturbed by acid-base imbalances.

It moves *in* when protoplasm grows and *out* when protoplasm disintegrates. It moves from cells to plasma under a variety of conditions all involving an excessive loss of NaCl and water from the body, i.e., hemorrhage, shock, adrenalectomy, intraperitoneal glucose solution, intestinal obstruction or fistulas. K moves into the blood during increased muscular activity or increased rate of metabolism and falls during rest or anesthesia. There is some evidence that it follows the carbohydrate cycle from muscle to liver and back again. It frequently rises and falls with the lactic acid level as in muscular exercise, hemorrhage, and asphyxia; likewise it frequently rises and falls with the sugar level, falling after insulin and rising (temporarily at least) after adrenalin. Possibly it moves from muscle to liver with lactic acid and from liver to muscle with sugar. To some extent potassium is under the control of endocrines being most specifically affected by the secretion of the adrenal cortex. In small concentrations potassium is excitatory and in larger concentrations it is inhibitory. In this capacity it is particularly effective and important in synapses or myoneural junctions. It causes a contracture of skeletal muscle and an increase of tone in heart and smooth muscle under proper conditions. Its effects are usually inhibited by Ca. Since K and Ca modify the most fundamental properties of protoplasm and cells they play a secondary modifying rôle in nearly every vital process. Since the number of ways in which a physiological reaction can be modified are very limited (usually limited to "increase" or "decrease") it is quite possible to classify artificially many drugs, hormones, poisons or other influences as K-like or Ca-like or parasympathetic and sympathetic with more apparent success than is really justified by the complexity of the situation.

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THE PORPHYRINS IN HEALTH AND DISEASE

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Few subjects have been the source of so much confusion as that of the porphyrins. Furthermore, few have been of such profound importance to medicine, since an understanding of the metabolism of the pyrrol compounds is essential to any clear conception of the normal or pathological physiology of the respiratory pigments, and these substances make up one of the most important systems of the body. Two sources of confusion have existed in the past: the complicated nomenclature applied to the porphyrins, and a lack of understanding by clinicians of the essential procedures for studying their metabolism. In view of this fact, whereas by convention it is proper in a review article to present all sides of a subject and to give impartial consideration to conflicting results of experimental work, this has not always been done in this communication. Any omissions, however, are due solely to a desire to simplify the presentation, and to the fact that many reports must be discarded because they lack accurate information concerning the types, kinds, and amounts, of porphyrins excreted. In an attempt to attain clarity, therefore, a general survey is presented, so simplified that it should be useful to those who have not been particularly familiar with the problems of pyrrol chemistry.

The chemistry of the porphyrins. Hemoglobin is a complex made up of a pigment of the pyrrol group known as protoporphyrin, combined with iron, and with a protein, globin. A chemically related, but artificial, product of hemoglobin disintegration, hematoporphyrin, was first produced in its crystalline form by Nencki and Sieber (111). It became apparent from the structure of this compound (Piloty, Küster, Willstätter and Fischer, 34) that it was not the porphyrin present in hemoglobin (protoporphyrin); that is, the pigment which would remain behind if iron and globin were removed. Kämmerer (72a) in 1924 however, isolated a porphyrin formed from blood by bacterial action. The work was continued by Fischer (34a), who with Schneller (34f)

and Lindner (34k) in 1925 identified Kämmerer's pigment as a protoporphyrin. Fischer and Zeile (35h), and Fischer, Treibs, and Zeile (35i) synthesized protoporphyrin in 1929 from simple pyrrols. They found that the synthetic iron-protoporphyrin compound was identical with the natural heme.

Salkowski (125), Garrod (41b), Stockvis (139) and others found porphyrins excreted in the urine in certain diseases. Garrod (41b) as well as Saillet (124), demonstrated the presence of small amounts of similar pigments in normal urines. They supposed that they had isolated hematoporphyrin since until 1915 investigators were unable to distinguish artificial porphyrins from the natural compounds. Prior to Fischer's work the term hematoporphyrin was used to designate any porphyrin excreted in the urine.

In 1915 Hans Fischer, working as a young chemist in the laboratory of Friedrich von Müller in Munich, was engaged in a study of the nature of the pigments in blood and bile. A patient named Petry, who has since become famous as the classical case of congenital porphyria, excreted large amounts of unidentified pigments. These were supposed to be responsible for the necrosis of his skin which occurred after exposure to sunlight. Fischer isolated two different pigments from the excreta of this case (34a, b, c). One of them, which he termed coproporphyrin, was found in both urine and feces, and the other, chemically different, called uroporphyrin, was found only in the urine. It is now known that small amounts of the same coproporphyrin are found in normal urine and feces, but that the uroporphyrin is a pathological product excreted almost exclusively in congenital porphyria.

This observation was the beginning of a series of studies of the relationship of the excreted porphyrins to the respiratory pigments, and to the products of their destruction. The type of uroporphyrin and coproporphyrin excreted by normals and in congenital porphyria was found to be fundamentally different from that of the protoporphyrin in hemoglobin. Later it was shown that two types of porphyrins are present in yeast also (34n). This simultaneous existence of two chemically distinct types of compounds was termed by Fischer the "dualism of the porphyrins" (34).

To understand this dualism the chemistry of the porphyrins must be considered briefly (34). The basic structure consists of 4 pyrrol rings linked together by 4 methene bridges, and is the porphin nucleus present in all porphyrins. It was synthesized in 1935 by Fischer and Gleim (35o), and independently by Rothemund (122) (fig. 1).

The hydrogen atoms numbered 1 to 8 in the formula (fig. 1) are replaced in the naturally occurring porphyrins by a series of substituting groups. For example, the protoporphyrin (fig. 2) present in hemoglobin and myoglobin has 4 methyl (1, 3, 5, 8), 2 propionyl (6, 7), and 2 vinyl (2, 4), groups substituted in place of the 8 hydrogen atoms in the basic porphin nucleus.

If the 8 hydrogen atoms are replaced by 4 methyl and 4 ethyl groups, 4 isomeric compounds can be derived, termed etioporphyrins types I, II, III and IV. They do not exist in nature, but are the basic structures to which the porphyrins are referred for purposes of identification. All the naturally occurring porphyrins so far analyzed have been found to correspond to etioporphyrins types I and III (fig. 3).

Porphin

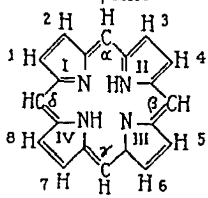
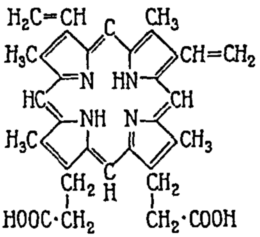


Fig. 1



Protoporphyrin
Type III

Fig. 2

Fig. 1. The structural formula of porphin
Fig. 2. The structural formula of protoporphyrin type III

If the 8 hydrogen atoms are replaced by 3 types of substituting groups, namely: 4 methyl, 2 propionyl, and 2 vinyl, compounds are obtained which are called protoporphyrins. Fifteen isomeric porphyrins of this kind are theoretically possible. The porphyrin in both hemoglobin and myoglobin, as well as the prosthetic group of other respiratory enzymes is protoporphyrin.

The vinyl groups of the protoporphyrins are transformed by mild reduction into ethyl groups, and in this way porphyrins are obtained which are called mesoporphyrins. For the 8 hydrogen atoms present in the porphin, 4 methyl, 2 ethyl, and 2 propionyl groups are substituted. Fifteen isomeric mesoporphyrins are theoretically possible. Mesoporphyrin was recently found in the feces by Zeile and Rau (161a). Fischer (34d) has reported the transformation of the protoporphyrin

present in natural heme into mesoporphyrin. Two different types of mesoporphyrin were isolated.

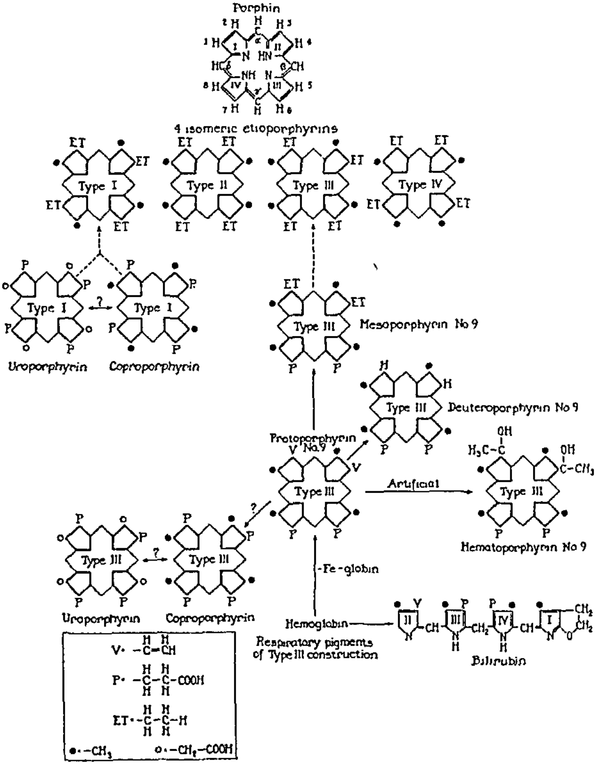


Fig. 3. The structural formulae of the porphyrins

If the 2 carboxyl groups in the mesoporphyrins are decarboxylated, etioporphyrins are produced which have 4 methyl and 4 ethyl groups

in place of the 8 hydrogen atoms. Only 4 isomers of etioporphyrin are possible, however (fig. 3).

Each of the 15 isomers of protoporphyrin and of mesoporphyrin corresponds to one of the 4 etioporphyrins. Fischer and his co-workers synthesized the 4 etioporphyrins and 12 of the 15 mesoporphyrins. He gave to the etioporphyrins the arbitrary numbers 1 to 4, and to the mesoporphyrins 1 to 15. Since he was able to transform the natural protoporphyrin into mesoporphyrin, and since its methyl ester was identical with the methyl ester of the mesoporphyrin number 9, the natural protoporphyrin is called protoporphyrin number 9 (fig. 3).

Since by decarboxylation of the mesoporphyrin number 9 an etioporphyrin is obtained which is identical with the etioporphyrin type III, the protoporphyrin number 9 is also a type III porphyrin. So far in nature only porphyrins have been found which correspond in their configuration to the etioporphyrin types I and III.

If the 2 vinyl groups present in the protoporphyrin number 9 are reduced and are replaced by 2 hydrogen atoms, a porphyrin is formed which is called deuteroporphyrin number 9 (fig. 3). This is also a type III porphyrin because it corresponds in its construction to the etioporphyrin type III. Fifteen isomeric deuteroporphyrins are theoretically possible. The deuteroporphyrin number 9 found in the feces under certain conditions and formed by bacterial removal of the vinyl groups of the protoporphyrin number 9 was first isolated by Watson (156a).

The hematoporphyrin number 9 produced from hemoglobin or hematin by Nencki is formed by the addition of 1 molecule of water to each of the 2 vinyl groups of the protoporphyrin number 9. It is an artificial compound, and has not been found in nature (fig. 3).

Under certain pathological conditions, a porphyrin can be isolated which has 2 propionyl groups in place of the 2 vinyl groups of the protoporphyrin number 9. It is called coproporphyrin. This compound has 4 substituted methyl and 4 propionyl groups, and 4 isomers are possible. If the 4 propionyl groups of the coproporphyrin obtained from protoporphyrin number 9 are decarboxylated, etioporphyrin type III is obtained. Hence the coproporphyrin is a type III compound (fig. 3).

The transformation of protoporphyrin number 9 into coproporphyrin type III may take place in nature but has not been effected in vitro. Coproporphyrin III was isolated first from a case of chronic porphyria by Hijmans v. d. Bergh and co-workers (61a), and was finally identified by Fischer and his associates (35f).

If the 4 methyl groups of the coproporphyrin type III are carboxylated a compound is formed which is called uroporphyrin type III. This compound was obtained first by Waldenström (155a) from the urine of a patient with acute porphyria. By decarboxylation uroporphyrin type III can be transformed into coproporphyrin type III (fig. 3).

The protoporphyrin number 9, the deuteroporphyrin number 9, the mesoporphyrin number 9, the coproporphyrin type III, and the uroporphyrin type III, are chemically closely related compounds. Hence a transformation of one into the other easily can be understood (fig. 3).

Fischer and his staff, Warburg and Negelein, Zeile, Stern, Schönheimer and others investigated the configuration of the porphyrins in the respiratory pigments (34). They proved that hemoglobin, (34, 35h, i), myoglobin (127), cytochrome C (161b), catalase (138), spyrographis-hemin (35g), and other pigments all contain porphyrins of type III configuration. Types other than III were not found in the respiratory pigments until the recent discovery by Fischer (34d) of a type I porphyrin in natural heme.

In 1929 Fischer (34, 35h, i) synthesized heme from simple pyrrols, which he combined into suitably constructed pyrromethenes. These were then condensed into a type III porphyrin and by further steps transformed into protoporphyrin number 9 (type III). By the combination of the latter with iron he obtained heme, which showed all the properties of the natural substance.

The exact steps of the natural synthesis are unknown, but it is assumed that it starts with very simple building stones. Fischer has suggested that possibly aceto-acetic acid may be one of the sources, and others have suggested proline, oxyproline, or tryptophane.

In the physiologic breakdown of hemoglobin bilirubin is formed (fig. 3), and is transformed by further reduction and oxidation into urobilin and urobilinogen, as well as into other bile pigments (34, 156). In the formation of bilirubin the porphin ring is opened in the alpha position and a chain compound results with a structure corresponding to type III porphyrin. Recent investigations (Lemberg, 83a, b, c; Barkan, 4a, b) indicate that under normal conditions the porphyrin ring is opened while it contains iron and while it is still combined with the protein molecule. It is important to note that in the normal transformation of hemoglobin into bile pigment no free porphyrin occurs.

Fischer isolated in 1915 from the feces of Petry a porphyrin which he called coproporphyrin because of its source, although it was later found in the urine also (34, 34a, b, c). He established the fact that this porphyrin is fundamentally different in its type from protoporphyrin

number 9 (type III) present in hemoglobin. As shown in figure 3, the coproporphyrin is similar in configuration to the etioporphyrin type I, and hence is a type I porphyrin. This compound was isolated from the urine and feces of normal human beings (Hoerburger, 64a; Watson, 156e; Dobriner, 23b; Fink, 33c), and of normal dogs (Dobriner, 23d).

If the 4 methyl groups in coproporphyrin type I are carboxylated a compound is formed which is called uroporphyrin type I. This is

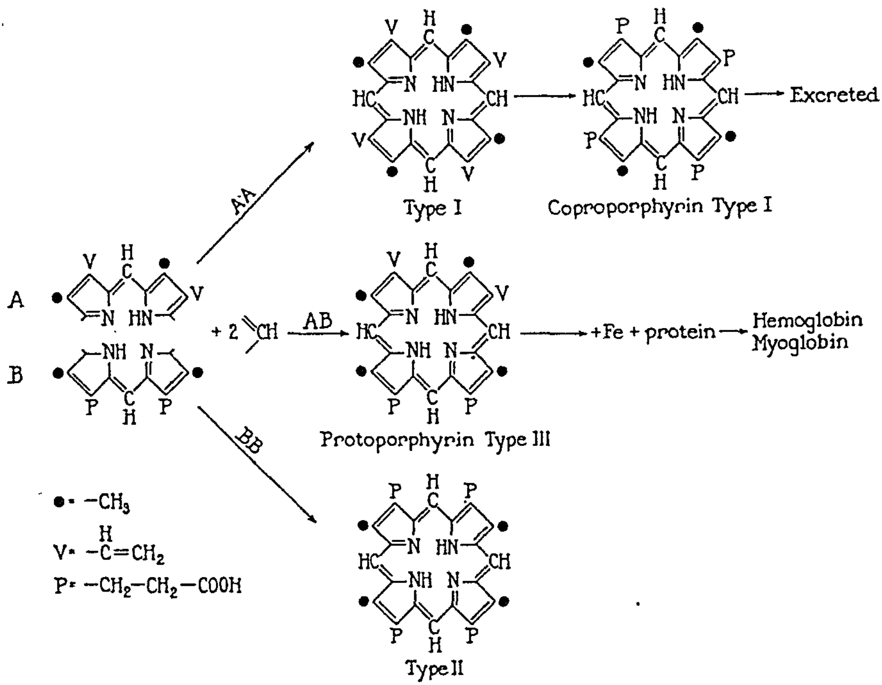


Fig. 4. A scheme representing the simultaneous synthesis of the type I and type III porphyrins.

present in large amounts in the urine of patients with congenital porphyria, and was isolated with coproporphyrin I by Fischer (34a, b, c).

It is important to realize that a transformation of one type of porphyrin into another by a change in the positions of the substituted groups is not possible chemically. It could be done only by degradation of the porphyrin complex to simple pyrrols, followed by resynthesis. There is no evidence so far that such a process occurs in human beings

or in animals under any circumstances. Hence Fischer suggested that the two types are synthesized independently (34).

In a study of the coexistence of two basically different porphyrin types, the so-called "dualism," Dobriner isolated and identified the types of porphyrins excreted in the urine and feces in over 100 cases of disease as well as in normal human beings and animals (23).

Early in the course of the studies by qualitative methods it was observed that there seemed to be a relation between increased hematopoietic activity and increased coproporphyrin I excretion. Quantitative studies were then begun. Finally a working hypothesis was formulated (23f, h, l, m) to explain the dualism. This explanation of the concurrent construction of types I and III porphyrins is represented schematically in figure 4.

As stated previously, porphyrins are synthesized by the combination of substituted pyrrols to form suitably constructed pyrromethenes, which are then condensed to porphyrins.

If one considers the two pyrromethenes A and B formed from substituted pyrrols, as joined together by two methene groups to form porphyrins, there are three possible combinations: 1, two molecules of A may unite, or 2, two molecules of B, or 3, one molecule each of A and B. As can be seen from the figure, the following compounds would form: (1) 1,3,5,7,tetramethyl 2,4,6,8,tetravinyl porphyrin, (2) 1,3,5,8,tetramethyl 2-4 divinyl-6-7 dipropionyl porphyrin, and (3) 1,4,5,8,tetramethyl 2,3,6,7,tetrapropionyl porphyrin. If the order of substitution is compared with the etioporphyrins in figure 3, it will be seen that A.B. is a type III, A.A. is a type I, and B.B. is a type II, porphyrin.

The individual compounds will be formed in relatively different amounts according to the conditions under which the synthesis operates, and to the relative amounts of A and B which take part.

The type II porphyrin which should be formed according to the outline presented has not yet been demonstrated in nature. This is due perhaps to its failure to be formed at all in the biological synthesis, or to the great difficulty of obtaining sufficiently large amounts of the compound to allow separation and identification.

Fischer tried to direct the *in vitro* syntheses in such a way as to obtain only one type of porphyrin, but in many instances mixtures of different kinds and types resulted ("mixed synthesis") (34).

In vivo, under normal conditions, the porphyrin synthesis is directed to the formation of relatively large amounts of type III porphyrins, and

small amounts of type I. The rate of production of type I seems to be in a definite ratio to the rate of formation of the type III compounds which later combine with iron and specific proteins to form respiratory pigments.

Under certain conditions there may occur an orderly increased or decreased formation of type III porphyrins associated with a simultaneous increase or decrease of type I production. Under other conditions this constant ratio may be disturbed with a resultant disorderly increased formation of type I compounds and often with the production of abnormal porphyrins as is the case in congenital porphyria.

This theory was first advanced in a preliminary communication concerning congenital porphyria in 1936 (23f), and experiments in human beings and animals were in progress at that time to prove its validity (23, b–o). Rimington independently advanced a similar hypothesis as an "enzymatic theory of hematopoiesis" (118a, c).

The isolation of porphyrins: their chemical and physical properties. Detailed descriptions of the chemical and physical properties of the porphyrins, with information regarding methods of analysis and synthesis, are found in the monograph of Fischer-Orth (34).

The porphyrins are of reddish color in acid solution, and in organic solvents show intense absorption bands in the visible region of the spectrum (Schumm, 130a; Fischer and Treibs, 35a; Kirstahler, 76). The position and number of the bands depend upon the pH and temperature as well as on the solvents, a subject investigated at low temperatures by Conant and Kamerling (19). The different kinds of porphyrins have absorption bands in different positions but the bands of isomeric compounds are in the same positions and therefore cannot be used for the identification of types.

In ultraviolet region of the spectrum the porphyrins show an intense absorption band in the region of 3900 to 4100 Å. They also show a very intense red fluorescence, as well as specific bands by fluorescence spectroscopy (Dhéré, 22; Borst and Königsdörfer, 12). In acid solution, the fluorescence may be detected in extreme dilutions and in this way minute amounts of porphyrins in organs and tissues may be detected. The relation of absorption to the constitution of the porphyrins is discussed in detail in the publications of Stern and Wendelein, Stern and Molvig, and Pruckner and Stern (literature, 34, p. 579). The relation of the intensity of fluorescence to the pH has been used for the identification of isomeric porphyrins by Fink and Hoerburger (33e, f).

Protoporphyrin, mesoporphyrin, deuteroporphyrin, and the copro-

porphyrins are all soluble in a mixture of acetic acid and ether, but uroporphyrin types I and III are not. Deuteroporphyrin and protoporphyrin are chloroform-soluble under certain conditions. Uroporphyrin type III is soluble in ethyl acetate, but uroporphyrin type I is not dissolved by this compound or by acetic acid. All porphyrins are soluble in strong mineral acids. The protoporphyrin, deuteroporphyrin, and hematoporphyrin form insoluble sodium salts and can thus be separated from the coproporphyrins. The ether-soluble porphyrins can be separated from ether by extraction with different concentrations o hydrochloric acid. The concentrations are called the specific HCl

TABLE 1

Physical constants of important porphyrins (34)

	HCl NO*		MELTING POINTS OF METHYL ESTER	SOLUBILITY OF FREE PORPHYRIN IN NaOH
	Free porphyrin	Methyl ester		
			°C.	
Protoporphyrin IX	2.0	5.5	228	Insoluble
Mesoporphyrin IX	0.5	2.5	216	Insoluble
Deuteroporphyrin IX	0.4	2.0	223	Insoluble
Hematoporphyrin IX	0.1		212	Insoluble
Coproporphyrin I	0.08	1.5	252	Soluble
Coproporphyrin III	0.09	1.5	135† / 170	Soluble
Uroporphyrin I		7 ca	302	Soluble
Uroporphyrin III		7 ca	256	Soluble

* The concentration of hydrochloric acid which will remove two-thirds of the porphyrin from an ether solution of the porphyrin when equal volumes are shaken together.

† Double melting point.

numbers (Willstätter). Some of the physical constants are given in table 1.

The coproporphyrins, uroporphyrins, deuteroporphyrins and mesoporphyrins, are identified as their methyl esters. Protoporphyrin undergoes a change of the labile vinyl groups during the processes of isolation and separation (Fischer, Treibs and Zeile, 35i), and it is reduced to mesoporphyrin for identification by the method of Fischer and Pützer (35b).

A detailed description of the methods used up to 1927 is presented by Fischer (34e), and since then Fischer and Duesberg (35m), Watson

(156f), Waldenström (155a, b), Dobriner (23b, k), and Rimington (118h) have reported modifications and improvements based on Fischer's procedures. The physical constants for the separation of the natural porphyrins have been described recently in the papers by Zeile and Rau (161a), and by Keyes and Brugsch (75, 15f). In table 2 a scheme for the separation of the fecal porphyrins is given which, with slight modifications, can be applied to those in urine (23k). For the isolation of uroporphyrin type I the method of Fischer and Duesberg (35m) should be used, and for uroporphyrin type III that of Waldenström (155a, b).

It has become increasingly important to separate different isomers. The methyl ester of coproporphyrin type III can be crystallized only with difficulty. Up to 1936 the usual method for identification of the important coproporphyrin was to recrystallize the methyl ester several times and thus raise the melting point. It seems certain that in many instances the presence of small amounts of isomeric compounds was overlooked. Dobriner (23b, c) introduced a simple method for the separation of the methyl esters of coproporphyrin types I and III. Rimington (118h) employs absolute ethyl ether instead of methyl alcohol.

For all measurements a quantitative separation of the porphyrins is essential. With the exception of a few conditions in which ether insoluble uroporphyrins are present, the acetic acid-ether method should be used. If proto-, deutero- or mesoporphyrins are to be determined, the patients should be on a hemoglobin- and myoglobin-free diet. The determinations of the content in either urine or feces should be made if possible on 3-day collections over a 9-day period. Many investigators in the past have only studied urine, but it seems to be essential to examine the feces also. Twenty-four hour collections of urine should be used because of the different concentrations of porphyrins excreted at different times. After isolation, purification, and separation of the different kinds of porphyrins, quantitative determinations may be made by spectroscopic (130a, 23k), spectrocolorimetric (17a, 156f), spectrophotometric (130a, g), photoelectric (23l), and fluorometric (152c, 15b, 32b) methods. The results of the determinations with the different procedures agree reasonably well, although fluorometric methods seem to give slightly lower values. Fluorometric procedures are essential for the determination of the protoporphyrin in blood.

The porphyrins in nature: It is not possible in this review to mention all the publications concerning this subject. The iron porphyrin com-

TABLE 2

A scheme for the separation, purification and identification of the porphyrins of feces

<table>
<tr><td colspan="4">I. Extraction of the total crude porphyrins with ether. Purification of total porphyrins</td></tr>
<tr><td colspan="4">II A. Saponification of the natural porphyrin esters with 20% NaOH. Separation of soluble and insoluble sodium salts</td></tr>
<tr><td colspan="2" rowspan="3">III a. Soluble sodium salts, coproporphyrins</td><td colspan="2">II b. Insoluble sodium salts, deuteroporphyrin and protoporphyrin</td></tr>
<tr><td colspan="2">II B. Separation of deutero- and protoporphyrins with 0.6% HCl from ether</td></tr>
<tr><td>0.6% HCl fraction, deuteroporphyrin (III B)</td><td>Ether fraction, protoporphyrin (III C)</td></tr>
<tr><td colspan="4">Purification</td></tr>
<tr><td colspan="2">III A. Coproporphyrins</td><td>III B. Deuteroporphyrin</td><td>III C. Protoporphyrin</td></tr>
<tr><td colspan="2">Extracted from ether with 0.2% HCl</td><td>Extracted from ether with 0.6% HCl</td><td>Remains in ether after extraction with 1.0% HCl</td></tr>
<tr><td colspan="2">Purification with chloroform and petroleum ether

Coproporphyrins in HCl</td><td>HCl concentration reduced to 0.2% and extracted with chloroform

Deuteroporphyrin in chloroform</td><td>Extracted with 5% HCl and dissolved in chloroform

Protoporphyrin in chloroform</td></tr>
<tr><td colspan="4">Identification</td></tr>
<tr><td colspan="2">IV A. As coproporphyrin methyl esters</td><td>IV B. As deuteroporphyrin methyl esters</td><td>IV C. As protoporphyrin or mesoporphyrin methyl esters</td></tr>
<tr><td colspan="2">V. Separation of coproporphyrin I and III methyl esters with methyl alcohol</td><td rowspan="4"></td><td rowspan="4"></td></tr>
<tr><td colspan="2">Methyl alcohol</td></tr>
<tr><td>Soluble</td><td>Insoluble</td></tr>
<tr><td>Coproporphyrin methyl ester III</td><td>Coproporphyrin methyl ester I</td></tr>
</table>

pounds and iron-protein-porphyrin compounds (respiratory pigments and enzymes) cannot be discussed, nor can the bile pigments. Excellent reviews have been written by Anson and Mirsky (2), McMaster (103), Fischer (34), and Watson (156).

Protoporphyrin number 9 (type III) is identical with Kämmerer's prophyrin, with Fischer's oöporphyrin, and with Schumm's hematerinic acid. It was the one obtained from the blood of Petry, who excreted a large amount of type I porphyrins. Recently Fischer (34d) announced the formation of mesoporphyrin numbers 2 and 9 from the protoporphyrin of natural heme. These are types I and III porphyrins respectively.

Protoporphyrin was produced by the bacterial fermentation of blood (Kämmerer, 72a), by the sterile autolysis of myoglobin (Hoagland, 63) and by treating reduced hemoglobin with concentrated hydrochloric acid. By treating hemochromogen with weak acids, the iron-protein part can be split off from the respiratory pigments with the production of free protoporphyrin (Schumm, 130a). If hemoglobin or myoglobin is digested in the intestinal tract, 89.5 per cent protohemin, 2.8 per cent protoporphyrin, 6.6 per cent deuterohemin and 0.9 per cent deuteroporphyrin, are formed (Haurowitz, 56a, b). The publications by Snapper (136), Schumm (130a) and Boas (11a, b) regarding the metabolism of hemoglobin in the intestine should be consulted. In the feces of normal human beings on a meat-free diet only traces of protoporphyrin are found. Among animals, rats excrete large amounts of protoporphyrin (118g) and it was isolated by Rimington from normal sheep liver (118b).

Fischer and Kögl (34j) isolated and identified protoporphyrin type III from the colored egg shells of different species of birds. Hijmans v. d. Bergh and Grotepass observed during the incubation of hen eggs the production of free protoporphyrin (61d). Its presence in cereals and other plant material (35c), as well as in fresh yeast, is reported by Fischer and co-workers (34n, 102) and by Schumm (130a).

Deuteroporphyrin number 9 (type III) is identical with Schumm's copratoporphyrin, and was isolated and identified from feces by Watson (156a). It is a disintegration product of the protoporphyrin of hemoglobin. Watson (156f) found related compounds which he called pseudo-deuteroporphyrins A and B. Fischer and Duesberg (35m) stated that they had isolated substances like deuteroporphyrin from the feces of rabbits, but never conclusively identified them as deuteroporphyrin, since they showed much lower melting points than deuteroporphyrin number 9.

Mesoporphyrin number 9 was isolated and identified recently by Zeile and Rau (161a), and Grotepass and Defalque (48e) from the feces of human beings.

The presence of coproporphyrin in biological material has often been reported. Identification of types was rarely possible. Fischer and co-workers reported the presence of coproporphyrin in seeds and plant material (34, 35c), and Kämmerer and Gürsching (72c) in foodstuff. In bacilli and fungi coproporphyrin is present in small amounts, as shown by Fischer and Fink (34n), Coulter and Stone (20), and Carrié and Mallinckrodt (17a, 17c, 94). Jakob recently isolated coproporphyrin type III from bacterial cultures (70b).

The presence of coproporphyrin I in urine and feces of normal and diseased individuals is discussed later in detail. Dobriner (23d, g) isolated it from the excreta of normal dogs. Fischer and co-workers (34, 34n), Fink (33a) and Mayer (102), reported the presence of coproporphyrin type I in yeast, and heme of type III construction was also identified. If yeast is poisoned with copper large amounts of coproporphyrin I are produced. Schonheyder (128) isolated coproporphyrin type I from eggs and followed the rate of its production during incubation. Coproporphyrin type III has been found in the urine of normal rabbits (Dobriner and Rhoads, 23j), and its presence in nature is reviewed by Rimington (118d).

Uroporphyrin type I is found in the urine of human beings and animals with congenital porphyria, but is not present in normal urine (Grotepass, 48d). Fischer (34), with Haarer (35l) and with Hofmann (35r), found it in mussel shells (pteria), and Waldenström (155a) confirmed this, but in two instances he found uroporphyrin which gave much lower melting points and different fluorescence curves. This suggests that the compound is intermediate between uroporphyrin types I and III. From the colored feathers of the bird Turacus, Fischer and Hilger (34i) isolated uroporphyrin type I. Turner (148a) found uroporphyrin type I in the skeleton of the fox squirrel.

Uroporphyrin type III was isolated by Waldenström (155a, c) from the urine of a large number of cases of acute porphyria. Völcker (154a, b), and independently Rimington (118c), isolated uroporphyrin III from many species of tropical bird feathers.

Bingold (8) described in the urine a pigment called pentdyopent, which can be artificially produced by the action of hydrogen peroxide on hematin or bile pigments, and Fischer and Müller (35s) identified it as a pyrromethene.

The physiology of the porphyrins. The photosensitizing action of

the porphyrins was discovered by Hausmann in 1910, and since then many experiments have been published. The reviews by Hausmann and Haxthausen (57a), Ellinger (28), and Blum (10a, b, c), should be consulted. There seems to be a difference in the action of the same porphyrin in different species (34, 35e). Hematoporphyrin has received special attention. The action of the naturally occurring porphyrins, protoporphyrin type III, coproporphyrin, and uroporphyrin types I and III, requires further study. Coproporphyrin type I sensitizes less than uroporphyrin type I, and the effect of uroporphyrin type III has not been studied. Coproporphyrin type III produces less light sensitivity than does type I (9b).

Hausmann and Kuen (57b) reported the effect of different kinds of porphyrins on the hemolysis of erythrocytes. "Light shock" after the injection of porphyrin was studied by Rask and Howell (116) and Smetana (135a, b, c), who also investigated the photo-oxidation of hematoporphyrin in the animal organism. In a famous experiment Meyer-Betz (106) injected himself with 200 mgm. of hematoporphyrin, and developed after exposure to light a severe edema of the skin of the exposed regions. The lesions are different from those of congenital porphyria, and light sensitivity continued for a considerable period.

Hinsberg and Merten (62c) observed a 50 to 100 per cent increase in the rate of protein metabolism after the injection of 4 mgm. of hematoporphyrin daily. The C:N ratio increased from 1.3 to 4.3, and the O:N ratio from 5.5 to 8.7. An increased excretion of chlorides was also present. Hinsberg and Ammon (62b) studied the influence of porphyrins on the auto-oxidation of fatty acids.

Gaffron (40), Boyd (13), Smetana (135a, b, c), and Holden (66a, b), observed that porphyrins combine with proteins (fibrinogen, albumin, and globin). Light hydrolyzes the proteins in the presence of porphyrin, and oxygen is necessary for this reaction. Gildemeister (44) employed cataphoresis in the study of the combination of serum proteins with porphyrins. Hematoporphyrin number 9, as well as coproporphyrin types I and III, combine with the albumin fraction but uroporphyrin types I and III do not do so. These findings are of great interest since Shibuya (133), as well as Rask and Howell (116), found that serum protects against photosensitization (57a).

The destruction of the porphyrins in tissues has been the subject of several studies. Sumner (142) observed that hematoporphyrin is destroyed *in vitro* by liver. Perutz (114) mixed liver with the urine of rabbits poisoned with sulfonal. This urine was high in its content of

porphyrin. Porphyrin could not be recovered from the mixture. Similar experiments were performed by Schreus and Carrié (129b) by adding liver tissue to the concentrated urine of patients with acute porphyria. The destructive effect could be abolished by heating the tissue to 56°C. Hijmans v. d. Bergh and co-workers (61b) perfused liver with solutions of protoporphyrin in defibrinated blood. The bile was shown to contain coproporphyrin, but none was formed when protoporphyrin was absent from the perfusion fluid. Coproporphyrin so produced can be only of type III configuration.

Little is known concerning the metabolism and quantitative excretion of the naturally occurring porphyrins, when administered parenterally or orally. This is due to the fact that accurate quantitative methods for porphyrin separation and determination in feces have been made available only recently. Fischer showed that, in rabbits, injected coproporphyrin type I is excreted in both the urine and the feces, but that uroporphyrin type I is only excreted in the urine (34). Vigliani (153f) observed that from patients with biliary fistulae only about 50 per cent of coproporphyrin type I and 20 per cent of type III injected intravenously could be recovered. Dobriner (23d), in similar experiments in dogs, recovered about 70 per cent of coproporphyrin type I in 24 hours. Fischer (34k), Schumm (130d), and Watson (156j), recovered an increased amount of coproporphyrin in the urine after administering protoporphyrin. The fate of hematoporphyrin has been investigated in more detail, since that compound has been used in the treatment of manic-depressive psychoses (68, 39). Neubauer (112) after injection recovered this compound from the bile. Similar experiments in animals are reported by Hutschenreuter (69), Jakob (70a), and Smetana (135c), and in human beings by Häcker (51), Jakob (70a), and Schumm (130g). All the investigators agree that only traces of injected hematoporphyrin are found in the urine. Grotepass and Hulst (48b) perfused kidneys and observed that uroporphyrin is excreted rapidly, coproporphyrin less so, and protoporphyrin not at all. Schumm (130a), Derrien and Cristol (21), and Kapp (73a), reported the presence of complex zinc and copper salts of porphyrin in the urine.

Fischer (34c) suggested that the toxic action of the porphyrins depends upon the number of carboxyl groups. In white mice the order of toxicity, as judged by the severity of the "light shock" produced, is: hematoporphyrin number 9, uroporphyrin type I, deuteroporphyrin number 9, coproporphyrin type I, and protoporphyrin number 9.

Coproporphyrin type III seems to be slightly toxic and uroporphyrin type III non-toxic.

The action on the intestinal tract of hematoporphyrin number 9, coproporphyrin type I, and uroporphyrin type I, was studied by Reitlinger and Klee (117), Supniewski (143), and Vannotti (152b). These compounds increased the tonus and the effect was not abolished by atropine. The action of porphyrins on the circulation was studied by Supniewski (143), Rask and Howell (116), Smetana (135a), and Vannotti (152b).

The impregnation with uroporphyrin type I of the bones and teeth in congenital porphyria has suggested experiments in animals. It is important to note that from cases of acute porphyria excreting large amounts of uroporphyrin type III, neither Waldenström (155a) nor Mertens (105c) could isolate that substance from the bones. Fraenkel (37), Hammer (54), Borst and Königsdörfer (12), Fikentscher (32d), and others found that in the bones of embryonic, as well as newborn, human beings and animals small amounts of porphyrin are present, chiefly in the zones of calcification. In experiments with growing animals these authors, as well as Fikentscher et al. (32g), followed the fate in the bones of injected porphyrin. Uroporphyrin type I is easily and quickly deposited, as is hematoporphyrin number 9 if injected in large amounts. Coproporphyrin type I, mesoporphyrin number 9, and protoporphyrin number 9, seem to have little or no tendency to deposit. Bingel (9b) proved that uroporphyrin type III is deposited in bones and teeth. Emminger and Battestini (30a) studied the bones of animals with lead poisoning, and found that they contained none of the coproporphyrin type III excreted. The rate of elimination of different porphyrins seems to vary. The bones of the fetus are not impregnated with the porphyrins injected into the mother (12, 37, 54).

Van Leersum (151) described a therapeutic effect of injecting hematoporphyrin into rats with rickets, but Emminger and Büchele (30b), and Marique (96), could not confirm this observation. The natural porphyrins have not been studied in this regard. Strauch (140) discusses rickets in children and its relation to porphyrins.

Hinsberg and Rodenwald (62d) reported experiments concerning the action of hematoporphyrin and protoporphyrin on the hypophysis of frogs, rats, rabbits, and dogs. An increased excretion of melanophore-dispersing hormone was observed. In the serum of injected animals a substance was found which inactivated the hormone, and the authors

believe that the inactivating agent is the one found in the serum of patients with cancer.

Hinsberg (62a) reported recently that after the injection of hematoporphyrin and protoporphyrin in infantile mice, premature follicle formation in the ovaries takes place.

Porphyrins in normal human beings. Coproporphyrin is always present in small amounts in the urine, and was identified by Hoerburger (64a), Fink and Hoerburger (33g), and by Fink (33c), as coproporphyrin I, a finding confirmed by Watson (156e), by Dobriner (23e), and by Zeile and Rau (161a). Watson's and Fink's results suggest the presence of small amounts of coproporphyrin III. Grotepass (48d) isolated from 10,000 liters of pooled normal urine a mixture of equal parts of coproporphyrin types I and III. The writers believe that whereas traces of type III may be present, they have not succeeded so far in identifying the compound from normal urine. Pooled urine may have included specimens from individuals suffering from disorders marked by the excretion of the type III compound. Grotepass did not find any protoporphyrin or uroporphyrin in the urine of normal individuals.

In the feces the coproporphyrin found by Fischer and Schneller (34f) by spectroscopic methods was isolated and identified as type I coproporphyrin by Watson (156f), and by Dobriner (23b). Coproporphyrin III so far has not been isolated from the feces of normal individuals, but some of the melting points reported suggest that possibly traces of this substance may have been present. Watson (156a) isolated deuteroporphyrin number 9 from the feces of a patient taking meat.

Watson (156f) isolated and identified the coproporphyrin present in bile as type I. Vigliani (153f) did not find any protoporphyrin in the bile.

In table 3 the amounts of porphyrin contained in normal urine are listed as reported by different investigators. The results obtained by the different methods correspond closely. Determinations of coproporphyrin in the feces have been reported by only a few workers, and it is to be deplored that in the extensive early work of Brugsch (15b, c, d) the porphyrins isolated were not differentiated at all. Vigliani and co-workers (153e, f, g) made rough estimates of the ratio between excreted coproporphyrin and protoporphyrin. Dobriner and co-workers (23b, l) measured the excretion of coproporphyrin in the urine and feces of normals for 9-day periods. The results are given in table 4. The total daily urinary and fecal excretion by adults varied between

306 and 376 micrograms, with an average of 350 daily. Brugsch (15a) states that normals excrete daily from 150 to 300 micrograms. Children appear to excrete less coproporphyrin than do adults (Dobriner et al., 23 l).

TABLE 3

The average daily excretion of coproporphyrin in the urine of normal individuals as determined by different investigators by different methods

AUTHORS AND REFERENCES	URINARY COPROPORPHYRIN LEVEL FOR 24 HOURS	METHODS APPLIED
	mcg.	
Franke and Fikentscher (38b)	10–30 (60)	Fl.
Hijmans v.d. Bergh et al. (61b)	10–100	Fl.
Brugsch (15a, b)	4–50 (80)	Fl.
Tropp and Siegler (147a)	18–110	Fl.
Vigliani et al. (153e)	6–46	Fl.
Vannotti (152a, c)	10–80	Fl.
Schreus and Carrié (17a)	0–60 (80)	Sp.col.
Lageder (79a)	0–100	Sp.col.
Thiel (145a, b)	0–100	Col.
Dobriner et al. (23b, k, l)	41–120 (143)	Ph.el.col.

Fl. = fluorimetric; Sp.col. = spectro-colorimetric; Ph.el.col. = photoelectric colorimetric; Col. = colorimetric.

TABLE 4

Quantitative coproporphyrin I excretion in micrograms in average daily values for 9-day period

CASE NUMBER	AGE	SEX	R.B.C.	HGB. PER 100 CC.	COPROPORPHYRIN		
					Urinary	Fecal	Total
			millions	*grams*	γ	γ	γ
1	12	F	4.5	15	35	191	226
4	21	M	4.6	13	87	231	318
2	25	M	4.5	15	123	205	328
5	33	M	4.59	14.4	102	274	376
3	35	M	5.08	15.9	121	241	362
6	72	M	4.3	14.8	64	242	306

The presence of protoporphyrin in blood was determined quantitatively by Hijmans v. d. Bergh (61b), who found that 8 to 12 micrograms are present in 100 cc. of normal blood. Findings in normals and in disease are reported by Lageder (79b), Vigliani and Angeleri (153b),

Vannotti (152b), and recently by Schumm (130g), who found average values of 13 to 15 micrograms per cent. Watson and Clarke (156h) demonstrated protoporphyrin in the reticulocytes, and Grotepass (48c) identified that in erythrocytes as protoporphyrin number 9 (type III). In the serum under normal conditions coproporphyrin does not seem to be present (Schumm, 130g).

The presence of protoporphyrin, and possibly of small amounts of other porphyrins, in the bone marrow of normal human beings and in pathological conditions is described by Borst and Königsdörfer (12). The megaloblasts and erythroblasts seem to contain a relatively large amount of porphyrin.

Günther (50a) detected traces of coproporphyrin in meconium and it was identified by Waldenström (155a) as type I. Fikentscher (32c) found coproporphyrin in the amniotic fluid. Since injected porphyrins do not pass through the placenta (54, 37) it must be assumed that the embryo synthesizes coproporphyrin type I. Fikentscher (32d) investigated the content of coproporphyrin in the serum of embryos and newborn infants. It was present in the last month of pregnancy, and at birth. Herold (60a) studied the excretion of coproporphyrin by newborn infants with icterus neonatorum.

No increase of the rate of excretion of coproporphyrin during normal pregnancy was found by Carrié and Herold (17b), by Herold (60b), or by Fikentscher (32e). In toxemia of pregnancy and in hyperemesis gravidarum an increase was observed.

Porphyrias. These conditions make up a group of congenital diseases marked by a disturbed metabolism of pyrrols and by the excretion of large amounts of porphyrins. The etiology is unknown. Günther (50a, b) classified the different sub-groups according to their clinical manifestations; Micheli and Dominici (107), and Carrié (17a), on a different basis. Waldenström (155a) employed Günther's terminology, and it will be followed in this paper. Three types of disorder have been described: 1. Congenital porphyria (Günther). 2. Acute porphyria (Günther-Waldenström). 3. Chronic porphyria.

Great sensitivity to light and the excretion of large amounts of type I porphyrins are features of congenital porphyria. Acute porphyria is also a congenital disease, but the patients manifest no light sensitivity, and uroporphyrin type III is excreted. Cases of a type intermediate between the first two have been described by Günther as chronic porphyria, in which either type I or type III porphyrin is excreted. Porphyria must be distinguished from porphyrinuria, a

term which simply means the excretion of abnormally great amounts of porphyrins in the urine.

Congenital porphyria. Congenital porphyria was reviewed by Günther in 1922 and 1925 (50a, b), by Mason et al. (98), Vannotti (152a, b), Watson (156i), and by Turner and Obermayer (148c). The last authors discussed 86 cases of which 9 were considered to be doubtful. Dobriner and co-workers (23o, 113), reported chemical studies of 3 cases. A detailed report of the post mortem findings in the famous patient Petry is to be found in the monograph by Borst and Königsdörfer (12).

Congenital porphyria is a rare inborn error of metabolism (Garrod, 41a) possibly inherited as a Mendelian recessive (Günther, 50a, b; Mackey and Garrod, 93, 41b). The classical manifestations are: 1, the excretion of large amounts of porphyrin; 2, discoloration of the teeth and bones by impregnation with uroporphyrin I; 3, sensitivity of the skin to light in the spring and summer, a symptom which often appears first in childhood. Blistering of the exposed areas of the face and extremities is observed, and the lesions heal with scar formation, followed in many cases by deformity of the affected tissue. Limited space precludes a discussion of the clinical manifestations, but certain symptoms of endocrine disturbance deserve mention. In many cases hirsutism was present—for example, in Hegler's and Fraenkel's "bearded lady" (59). Morphological changes of the endocrine glands of Petry were described by Borst and Königsdörfer (12).

The disease ochronosis in animals was discovered by Schmey (126) in 1913 to be congenital porphyria. A review of the literature concerning this disorder is given by Fourie (36a), and by Fink (33b). Uroporphyrin type I was isolated from the bones of swine with porphyria by Fink and Hoerburger (33d). Fourie (36a) with Rimington (36b) reported congenital porphyria in living cattle.

The etiology of congenital porphyria is unknown. Dobriner and co-workers (23f, o), and Rimington (118a, c), independently advanced similar explanations of the disordered pigment metabolism, as follows: In congenital porphyria the normal ratio between the formation of type I and type III porphyrins is disturbed to give a disproportionate or disorderly type of synthesis in favor of type I. The disturbance is quite unlike any other disorder of pyrrol metabolism so far observed in human beings or in animals.

The relation of the porphyrins to light sensitivity is discussed elsewhere, and the fact is stressed that the different types and kinds of

porphyrins vary considerably in their photosensitizing action. In congenital porphyria Borst and Königsdörfer (12) and Carrié (17a) obtained evidence by fluorometric methods that porphyrins are deposited in the skin. Experiments with animals indicate that uroporphyrin type I, when injected in pure solution, sensitizes to light. Many investigators have attempted to produce artificially the skin lesions of congenital porphyria by exposing the patients to light from artificial sources. No successful experiments are reported. The literature is reviewed by Perutz (114), Günther (50a, b), Hausmann and Haxthausen (57a), Carrié (17a), Gottron and Ellinger (46a, b), and by Blum (10a, b, c). It has been suggested that in congenital porphyria the excretion of a brown pigment of nonporphyrin nature caused the disorder, since if this pigment is injected into animals simultaneously with type I porphyrins, no light sensitivity occurs (34h). Many studies have been made of the effect of hematoporphyrin number 9 on human beings and animals, but no condition like congenital porphyria has resulted. No experiments have employed uroporphyrin type I combined with coproporphyrin type I, the compounds which are excreted in congenital porphyria.

The early investigators often did not distinguish between congenital porphyria and hydroa aestivale or vacciniforme. In 1937 Mathews (100) collected 57 cases of hydroa aestivale; of these patients the urine of only 32 was examined for porphyrin. It was present in 22, but no clear evidence proves that these cases were true congenital porphyria. The authors recently studied a severe and typical case of hydroa vacciniforme. The urine and feces contained a normal amount of coproporphyrin type I (23e), and no abnormal kinds of porphyrins could be detected, a finding which is in agreement with Günther's (50a) early report. It should be emphasized that hydroa aestivale and vacciniforme are only symptom complexes and that congenital porphyria with similar skin lesions is a well-defined disorder of pyrrol metabolism.

The urine excreted in congenital porphyria is of a Burgundy red color, with a brown tinge, the latter due to a pigment isolated by Fischer et al. (34h). It contains large amounts of coproporphyrin type I and uroporphyrin type I, as proved first in 1915 by Fischer (34c). In many cases the urine darkens considerably after exposure to light, a phenomenon which is due to the photo-oxidation of leucoporphyrins. The facts concerning the pigments excreted in the urine of 9 patients are summarized in table 5. In 1936 Fischer and Libowitzky (35p) isolated from a large amount of uroporphyrin type I a small amount of

type III compound, and in 1939 Mertens (105d) found in the coproporphyrin fraction of the same patient much type I and a small amount of type III. The melting points reported by other investigators indicate the presence in their material of small amounts of isomers.

TABLE 5

The melting points of the methyl esters of the porphyrins excreted by the reported patients with congenital porphyria

	CHEMICAL WORK UP	REFERENCE	CLINICAL WORK UP	REFERENCE	MELTING POINT OF PORPHYRINMETHYLESTERS, °C.					
					Urine				Feces	
					Uroporphyrin		Coproporphyrin		Coproporphyrin	
					Type I	Type III	Type I	Type III	Type I	Type III
1	Fischer	34a, b, c	Günther Weiss	50a, b 158	293		250		249	
	Fischer-Hofmann	352	Borst-Königsdörfer	12	302	261				
	Mertens	105d	"Case Petry"						252	206*
2	Fischer-Zerweck	34	"Case Molzberger"	34	275				247/51	
3	Allot	93	Mackey-Garrod	93	283				248	
4	Allot	3	Ashby	3	286				245	
5	Jost	71	Schmidt-La Baume	71	272					
6	Waldenström	155a	"Case Schlosser"	155a	289		245		245	
7	Dobriner et al.	23o	Peachey et al.	113	286		250		249	
8	Dobriner et al.	23o	Guild	Not pub.	285		251		250	
9	Dobriner et al.	23o	Hardgrave	Not pub.	279		249		241	
10‡	Rimington et al.	118a, i	Fourie	36a, b	293	260	233/35	138† 172	250	
Melting points of the analytic methylesters (34)					302	261	252	135† 170	252	206*

* M.P. of Cu. methylester.
† Double melting point.
‡ In cattle.

In the feces of two patients Dobriner et al. (23o) found only traces of protoporphyrin and deuteroporphyrin; from one a conjugated coproporphyrin I was isolated.

The presence of coproporphyrin in the serum was observed by several investigators (Fischer, 34; Schumm, 130a). The hemoglobin of Petry contained normal protoporphyrin number 9, a type III compound. Fischer (34c) and Schumm (130b) reported some quantitative studies of the amount of coproporphyrin and uroporphyrin excreted by

Petry, and similar studies of two children are recorded by Dobriner and co-workers (23o).

Fischer and co-workers (34m) isolated and identified uroporphyrin type I from the bones and kidneys of Petry, as well as coproporphyrin type I from the bile, which contained no uroporphyrin. Spectroscopically, uroporphyrin and coproporphyrin were present in the bone marrow, liver, and kidney. In the blood plasma and in the intestine, coproporphyrin but no uroporphyrin, was seen spectroscopically.

Duesberg (25b) in 1931 suggested the treatment of congenital porphyria with liver extract. Schreus and Carrié (77a), as well as Vannotti (152b), published evidence that the administration of liver extract decreased the rate of porphyrin excretion. Dobriner and co-workers proved that the injection of large amounts of crude liver extract (230, 113), or ascorbic acid (239), caused a considerable decrease of the content of coproporphyrin and uroporphyrin in the excreta.

Rimington (118a), and Rimington, Roets, and Fourie (118i), in their unique studies of porphyria in cattle, isolated from the urine uroporphyrin Type I and small amounts of Type III, as well as coproporphyrin I and small amounts of Type III from urine and feces. One cow excreted daily up to 168 mgm. of uroporphyrin and 90 mgm. of coproporphyrin in the urine, as well as 1,000 mgm. of coproporphyrin in the feces. The ratio of coproporphyrin Type I to Type III in the urine was roughly 28 to 1. Uroporphyrin Type I was isolated from the bones, the bone marrow, spleen, and liver, and was seen spectroscopically in the red blood cells and kidney. It could not be found in the bile nor in the blood plasma. Coproporphyrin Type I was isolated from the bone marrow, spleen, bile, red blood cells, and blood plasma, and was absent in the bones and in the liver.

Fink (33b), and Fink and Hoerburger (33d), reported the isolation of uroporphyrin type I from the bones of pigs with congenital porphyria, and a morphological study is reported by Fikentscher (32a).

Acute porphyria. This condition is a clinical and metabolic entity reviewed by Günther (50a, b). Other cases were reviewed and described by Mason and co-workers (98), and by Vannotti (152b, d). A detailed description of the chemical and clinical findings in this disorder is given by Waldenström in his monograph (155a). A review of the cases in which the types and kinds of porphyrins excreted were determined, and with the chemical findings in three new cases, was published by Mertens (105c).

Cases of acute porphyria do not show light sensitivity and do excrete

large amounts of uroporphyrin type III, chiefly during the acute stage. Waldenström first identified the uroporphyrin excreted as type III compound (155a, b). So-called acute porphyria is in reality a chronic metabolic disorder, and the name was applied because of the fact that large amounts of porphyrins were excreted during the abdominal or paralytic attacks. Waldenström reviewed 179 cases, of which 103 were his own. Since that time others have been reported by Mertens (105c), Dobriner (23a, c), and Turner (148b). Clinical reports were published by Magendanz (95), Bingel (9a), von Drigalski (24), Minnibeck and Strasser (108), Zorn (163), Eldahl (26), Kurt (78), and Geissler (42).

The disease is more common in women than in men, as shown by Waldenström's geographically uniform material. It is familial, and has been observed in three generations. It is inherited as a Mendelian dominant. No single case of "congenital porphyria," was observed in any of the acute porphyria families (155a). The clinical picture varies greatly. The classical symptoms are of three types: 1, abdominal; 2, nervous; 3, mental. All three may appear independently or together. The abdominal symptoms are marked by severe cramp-like pain, vomiting and constipation; often with slight fever, hypertension, and tachycardia. Frequently the acute attacks seem to be associated with menstruation, and cases have been described in which they occurred immediately post partum (155a, 78, 163, 92). The nervous symptoms consist of a symmetrical progressive paresis, sometimes with neuritis. It is often termed Landry's paresis, but Waldenström points out that the classical ascending type of Landry's disease is found only in exceptional cases. The mental cases may present any type of psychic disturbance.

The etiology of acute porphyria is still unknown. Fischer, Waldenström, and Vannotti, suggested that myoglobin might be the source of the uroporphyrin, but this idea has been generally abandoned. Some relation to a disturbed production or breakdown of any of the respiratory pigments is theoretically possible. Waldenström suggested that the disease is a constitutional reaction to toxic factors (155a). No alterations of hematopoiesis are recorded in acute porphyria, but in several cases disturbed liver function, calcium and cholesterol metabolism were observed (1, 101, 155a).

Barker and Estes (5), as well as Vannotti (152b), and others, observed the sudden development of symptoms of Graves' disease in a number of cases. Magendanz's case had an obesity of the Fröhlich type (95); changes in the hypophysis and the adrenals have been described (92); Grünwald (49) reported a virgin who secreted cholestrum, and Harbitz

(55) a man with swelling of the breasts during an attack. Mason et al. (98) reviewed the morphological studies of the disease, and described a lesion of the coeliac ganglion.

In the urine large amounts of uroporphyrin are found. Löeffler (89) isolated a compound with a melting point of 262°C, Fischer and Duesberg (35m, n) of 269°C, Dobriner (23a), of 253°C, Hoerburger and Schulze (64c) of 289°C, and Turner (148b) of 275–278°C. These figures suggest that the pigment excreted is not the uroporphyrin type I found in congenital porphyria. The type I compound of Petry melted at 293°C.

Waldenström (155b), with Fink and Hoerburger (155c), and Waldenström (155a), identified the pigment as uroporphyrin type III. Mertens (105c) confirmed Waldenström's findings and in two cases obtained from a large amount of uroporphyrin type III a small amount of type I. The findings of the authors mentioned suggest that much type III and varying amounts of type I were excreted.

Weiss (158), Waldenström, Fink and Hoerburger (155e), and Waldenström (155a), isolated from the feces coproporphyrin type I, and the last author in one case coproporphyrin type III. Dobriner (23c) isolated coproporphyrin of both types I and III, a finding which Mertens (105c) confirmed in two cases.

The types of porphyrin excreted in acute porphyria are tabulated in Waldenström's (155a) and Merten's (105c) publications. Quantitative determinations were made by Günther (50a), Löeffler (89), Roth (121b), Hoesch and Carrié (65), Kurt (78), and Vannotti (152b).

One patient excreted up to 260,000 micrograms of uroporphyrin daily, and a small amount of coproporphyrin (89). In other instances up to 30,000 micrograms of uroporphyrin are excreted daily in the urine and variable amounts of coproporphyrin in the urine and feces.

Mertens (105c) and Magendanz's (95) patient excreted daily in the urine before a nervous attack 2,000 micrograms: during the attack 50,000 micrograms, and after the attack 11,500 micrograms of uroporphyrin type III, as well as 600 micrograms of unidentified coproporphyrin in the feces. The patient of Mertens (105c) and Bingel (9a) excreted 10,000 micrograms of uroporphyrin type III, 1,100 micrograms of coproporphyrin type III in the urine as well as 300 micrograms of a mixture of coproporphyrin types III and I in the feces. In the serum traces of coproporphyrin and uroporphyrin were present.

Waldenström (155a) and Mertens (105c) could not find any uroporphyrin type III in the bones of patients with acute porphyria. This

is remarkable because the uroporphyrin type I is deposited in relatively large amounts in the bones in congenital porphyria.

The color of the urine during the attacks of acute porphyria is usually red, but in certain cases Waldenström observed a straw-yellow color which darkened considerably after exposure to light. Waldenström (155a), Waldenström and Wendt (155g), and Waldenström with Vahlquist (155f, 150), isolated the chromogens present in the urine of patients with acute porphyria and termed them porphobilin and porphobilinogen. On boiling those substances at an acid reaction, they found small amounts of ether-insoluble porphyrins, possibly uroporphyrin type III. They suggest that the chromogens are two different isomers of dipyrreltetracarbonic acid. A further investigation of the nature of the chromogens in one case was published by Sachs (123).

Chronic porphyria. This term was applied by Günther to certain cases marked by an increased excretion of porphyrin and which could not be classified as either congenital or acute porphyria. Waldenström designated these cases "porphyria cutanea tarda." The skin of the patients is somewhat sensitive to light, and symptoms referable to the intestinal tract are present. The chemical findings, like the clinical manifestations, differ from those of classical cases of congenital and acute porphyria.

Hijmans v. d. Bergh and his co-workers (61a) isolated large amounts of coproporphyrin type III from the excreta of one case. The pigment was identified by Fischer and co-workers (35f). Fischer and Duesberg (35 m), isolated coproporphyrin type III from the urine. Hijmans v. d. Bergh and Grotepass (61c) published a case with renal insufficiency and no copro- or uroporphyrin in the urine. Large amounts of coproporphyrin type I were in the feces and the serum. Grotepass and Delfaque (48e) reported a patient who excreted in the urine small amounts of coproporphyrin type I and III, with no uroporphyrin. From the feces large amounts of coproporphyrin types I and III were isolated, as well as mesoporphyrin number 9 (type III), and protoporphyrin number 9 (type III). No deuteroporphyrin was present. From the case of Gottron and Ellinger (46b), Fischer and Duesberg (35m) isolated uroporphyrin type I and III, and from another case uroporphyrin types I and III, and coproporphyrin type III. From the urine of another patient Hoerburger and Schulze (64c) isolated uroporphyrin type I and coproporphyrin type III. From the urine of the patient of Urbach and Blöch (149), Waldenström isolated uroporphyrin type III (155a). Uroporphyrin types I and III and very small amounts of

unidentified coproporphyrin were obtained from the urine of the case of Wendelberger and Klein (157). The case reported by Dobriner (23c) as chronic porphyria was one of acute porphyria with mental symptoms.

Porphyrins in febrile conditions. Huppert in 1887 reported an increased porphyrin excretion in fever, and Günther (50a, b) reported similar observations and reviewed the literature. Schumm (130c), Lageder (79a), and Tropp and Penew (147b) stated that patients with tuberculosis excreted an increased amount of coproporphyrin (35 to 582 micrograms daily). In no case was the type of porphyrin identified.

In lobar pneumonia Dobriner (23a) isolated coproporphyrin I from both the urine and the feces, and in one case complicated by jaundice a small amount of coproporphyrin III was obtained (23j). Increased amounts of coproporphyrin I were isolated from the urine by Dobriner (23a), Watson (156c), and Vigliani and Libowitzky (153d), from cases of lung abcess. In the febrile attacks of schizophrenic patients Libowitzky and Scheid (84) reported an increased excretion of coproporphyrin I. In induced fever, increased urinary porphyrin values were reported by Carrié (17a), Vannotti (152b), and by Brunsting and co-workers (16b). This increase was not regular, and could not be correlated with the degree of fever. Kapp and Coburn (73b) isolated coproporphyrin III from the urine of patients receiving pyramidon and salicylates for rheumatic fever, and this finding, if confirmed for untreated patients, is of great importance. (Cf. Brownlee's (14) report of pyramidon intoxication in rats.) The evaluation of these data is difficult, but there seems to be no doubt that frequently in febrile states increased amounts of porphyrin are excreted, at least in the urine. In certain instances, furthermore, there is a suggestion that the total output is higher than normal. So far almost no fecal determinations in this type of case have been reported; hence no definite conclusions can be drawn.

Quantitative studies of the porphyrin content of blood in febrile conditions are reported by Hijmans v. d. Bergh and co-workers (61b), who found up to 46 micrograms of porphyrin in 100 cc. of normal blood compared with from 2 to 12 micrograms in normals.

Roets (119) reported a study of the porphyrin metabolism of African cattle with East Coast fever. He isolated from the urine and feces coproporphyrin type III and small amounts of type I. From 100 grams of feces 94 to 104 micrograms of coproporphyrin were obtained

as against 14 to 25 micrograms in normal animals. The ratio of coproporphyrin type I to type III in the urine was 1:31, and in the feces 1:1.5.

Porphyrins in liver disease. Since the early investigators, Salkowski, McMunn, Garrod, and many others, observed an increase of the urinary porphyrins in liver disease, many theories have been advanced concerning this abnormality. We omit a discussion of them since most investigators did not distinguish between coproporphyrin types I and III. In most instances type I is excreted, but in certain cases in which no exogenous intoxication can be demonstrated coproporphyrin III is found. Frequently relatively large amounts of protoporphyrin have been detected in the feces.

The normal liver excretes porphyrins of type I in the urine and the bile, but coproporphyrin III is excreted nearly exclusively through the urine, and to what extent a disturbed liver function may influence the urinary:fecal ratio is unknown. The coproporphyrin excretion in the urine is thought to be a very sensitive indication of hepatic dysfunction, but the authors feel that studies of the urine only, without estimations of the fecal coproporphyrin and determination of the types excreted, do not give adequate information. Brugsch (15a, d) was the first to investigate the urinary:fecal ratio of porphyrin excretion and found it to be between 0.2 and 0.6 under normal conditions. With the methods used he determined coproporphyrin, protoporphyrin, and deuteroporphyrin together in the feces. Localio, Schwartz and Gannon (88), determined the relation of the urinary to the fecal coproporphyrin excretion in normals and in a number of cases of liver dysfunction. In the normals, ratios between 0.3 and 0.6 were observed, and in liver disease from 0.8 to 22.0. It appears that the ratio is of more significance for the diagnosis of liver disease than is the total output, the highest ratios being observed in cases with the most severe liver damage, as judged by clinical evidence.

Watson (156b) isolated coproporphyrin I from the urine of a patient with cirrhosis of the liver due to cinchophen. Dobriner (23a) isolated the same pigment from the urine of 18 patients with catarrhal jaundice, obstructive jaundice, atrophic cirrhosis, and lymphosarcoma of the liver. Vigliani and Libowitsky (153d) isolated coproporphyrin I from a case of cirrhosis.

Dobriner, (23a) isolated coproporphyrin III from the urine of patients with atrophic cirrhosis, hemachromatosis, and melanosarcoma of the liver. From one case of hemachromatosis an unknown porphyrin with a melting point of 222°C was obtained. Vigliani and Libowitzky (153d), isolated coproporphyrin III from a patient with cirrhosis as-

sociated with lues, but the patient had received mercury shortly before the study and this may have contributed to the result.

The isolation of coproporphyrin I was reported by Dobriner (23b), from the feces of patients with atrophic cirrhosis, with catarrhal jaundice and with obstructive jaundice. From the feces of one case of cirrhosis Vigliani and Libowitsky (153d) isolated coproporphyrin I. Dobriner (23b) isolated a mixture of coproporphyrin types I and III from the feces of a case of hemachromatosis which excreted type III in the urine. Zeile and Rau (161a) isolated mesoporphyrin number 9 from the pooled feces of patients with liver disease.

Brugsch (15a, d), Franke (38a), and Dobriner (23b), reported large amounts of porphyrin in the urine of patients with obstructive jaundice. Watson (156f) reported one patient with complete common duct obstruction who excreted no coproporphyrin in the feces, but protoporphyrin and deuteroporphyrin were present. In similar cases with biliary fistulae Vigliani (153f) observed low values for coproporphyrin in the urine, but when no fistula was present the levels were high.

In passive congestion of the liver Thiel (145a, b), Brugsch (15a, d), Dobriner (23a), and Kaunitz (74), observed a moderate increase of urinary coproporphyrin.

Tropp and Penew (147b) reported normal levels of porphyrin in the urine of patients with cholelithiasis and cholecystitis without icterus. In cholangitis Brugsch (15a, b) and Lageder (79a) reported increased levels in the urine (185 to 547 micrograms).

Günther (50a), Thiel (145a, b), Dobriner (23a), Lorente and Scholderer (90), Brugsch (15a, d), Franke (38a), Tropp and Penew (147b), Vigliani (153f), and Kaunitz (74), reported increased rates of excretion of coproporphyrin in the urine of patients with catarrhal jaundice (90 to 408 mcg.) daily. Brugsch (15a, d), Tropp and Penew (147b), and Dobriner (23a), observed during recovery a slow decrease to normal or slightly increased levels. In the blood Vigliani (153e) observed increased levels of the protoporphyrin content of the red blood cells (85 and 111 micrograms). He also found coproporphyrin in the plasma.

In acute and sub-acute yellow atrophy of the liver Günther (50a), Brugsch (15a, d), Franke (38a), and Dobriner (23a), reported excretions of between 180 and 800 micrograms daily.

In hepatic disease due to syphilis Lageder (79a) and Tropp and Penew (147b) reported daily urinary coproporphyrin levels of from 79 to 441 micrograms.

In cirrhosis of the liver Brugsch (15a, d), Lorente and Scholderer

(90), Franke (38a), Kaunitz (74), Lageder (79a), Tropp and Penew (147b) and Dobriner (23a) report 70 to 1250 micrograms of coproporphyrin daily. No suitable quantitative studies of the porphyrin content of the feces in this type of case have been reported.

In lenticular degeneration, Tropp and Penew (147b) observed a normal rate of coproporphyrin excretion in the urine. In a few cases of hemachromatosis Günther (50a), Eppinger (31a, b), Vannotti (152b), and Dobriner (23a), found high levels in the urine. Increased excretion in the urine of patients with metastatic tumors of the liver are reported by Günther (50a), Thiel (145a, b), and Brugsch (15a, d). Daily excretions of from 120 to 615 micrograms are reported by Tropp and Penew (147b), Vigliani (153f), Lorente and Scholderer (90), and Dobriner (23a).

Porphyrins in diseases of the blood. Pernicious anemia. As early as 1897 Taylor (144) found porphyrin in the urine of a patient with pernicious anemia; Günther (50a) reviewed the early studies and reported similar findings. Fischer and Zerweck (34g) found coproporphyrin in both urine and feces.

The coproporphyrin in the urine of patients in relapse was identified as type I in 1937 by Watson (156f), and by Dobriner (23e, h). Watson (156c, f) also identified the coproporphyrin in the feces of three patients as type I. Dobriner (23b), Dobriner and Rhoads (23g), with reports of seven cases, and Vigliani and Libowitsky (153d), confirmed the observation. Watson isolated from the feces of one patient a chloroform-soluble porphyrin of which the methyl ester melted between 189° and 191°C. This was somewhat similar to a porphyrin he isolated from a patient with hemolytic jaundice, and called pseudodeutero-porphyrin A. Increased amounts of protoporphyrin in the feces were found by Watson, Dobriner, Vigliani and Sonzini, and Vigliani and Libowitsky.

Fischer et al. (34m) found coproporphyrin in the bone marrow, and Borst and Königsdörfer (12) observed an increase in the porphyrin content of the megaloblasts and erythroblasts. Watson and Clarke (156h) proved that the increase of protoporphyrin during remission was due to the presence of that pigment in reticulocytes. Vannotti (152b) reports high protoporphyrin levels in the bone marrow with a high coporporphyrin level in one of the cases and a low one in the other. This finding was confirmed by Vigliani and Sonzini (153e), who also found that patients in relapse had a low protoporphyrin content of red cells and plasma, and that the values increased during the

early stages of remission. In relapse traces of coproporphyrin were present in the plasma.

Duesberg (25a), Thiel (145a, b), and Brugsch (15a, c), Lageder (79a), Lorente and Scholderer (90). Vigliani and Sonzini (153e), and Kaunitz (74), made quantitative studies and found somewhat increased levels of coproporphyrin in the urine of certain patients in relapse, whereas in others normal levels were obtained. A decrease in remission was found first by Duesberg (25a), and later by Brugsch (15a), Vannotti (23h), and others. Dobriner with Barker (23c), and with Rhoads (23h), observed three patients in relapse over long periods and found considerable variations (38–169 micrograms) in the daily output. Determinations of the amounts of porphyrins in the feces were made by Brugsch (15a, c), and by Vigliani and Sonzini (153e), but their values are for total porphyrins without quantitative separation of types and therefore cannot be taken as significant. A study of coproporphyrin in the feces was made by Watson (156f). High levels in relapse and low levels during remission were obtained.

The results of the quantitative studies of the urinary and fecal coproporphyrin excretion in relapse and remission in the 3 cases described by Dobriner and Rhoads, together with values for Watson's cases are given in table 6.

Dobriner and Rhoads (23h) employed the rate of excretion of coproporphyrin type I as an index of an orderly type of hematopoietic activity. Hence the findings in pernicious anemia suggested that the disorder was marked by active blood regeneration during relapse and less active during remission.

Hemolytic jaundice. The coproporphyrin excreted in the urine of seven patients with hemolytic jaundice was identified as type I by Dobriner (23a). Two of the cases were of the familial type and five of the acquired. From one familial and one acquired case coproporphyrin type I was isolated both before and after splenectomy. Watson (156e) also isolated coproporphyrin type I from the urine in a case of the acquired disease.

Watson (156e, f), from the feces of 6 patients with acquired and one with familial jaundice, isolated coproporphyrin type I. The same porphyrin was obtained by Dobriner (23a, b, m) from both the urine and the feces of 5 patients. Protoporphyrin was found in increased amounts in the feces by Watson and by Dobriner. Watson isolated and described the properties of a chloroform-soluble porphyrin obtained from the deuteroporphyrin fraction, and which could not be identified.

The melting point of the ester was between 202° and 203°C. He called this compound pseudo-deuteroporphyrin A; it may be identical with that described by Schumm (130f) as saproporphyrin.

The literature contains reports of variable results of quantitative studies of coproporphyrin in the urine of patients with hemolytic jaundice. Günther (50a), Fischer and Zerweck (34g), Duesberg (25a), Lageder (79a), Vigliani and Sonzini (153e), and Vannotti (152b), found the content generally low. Dobriner (23a), in four cases of acquired and two cases of familial hemolytic jaundice, reported levels higher than normal. In one patient with a severe hemolytic crisis of the acquired disease, as much as 2,500 micrograms daily of urinary coproporphyrin were excreted, whereas in a previous period of observation no more than 800 micrograms per day had been obtained. In another unpublished case the average daily excretion over a 15 day period was 211 micrograms in the urine and 441 in the feces. Watson found 1,020 micrograms in the feces of one patient (156f).

TABLE 6

The average daily excretion of coproporphyrin I and of urobilinogen in relapse and remission of pernicious anemia and hemolytic jaundice

CASE NUMBER	COPROPORPHYRIN TYPE I (MCG.)						UROBILINOGEN (MGM.)		
	Urine		Feces		Total		Relapse	Remission	Reference
	Relapse	Remission	Relapse	Remission	Relapse	Remission			
1	158	42	474	130	632	172	442	93	(23g)
2	138	66	474	282	612	305	900	150	(23g)
3	133	95	345	105	478	200	478	262	(23g)
4			520	260					(156b)
5			570	240					(156b)
	a	b	a	b	a	b	a	b	
*	318	81	401	290	718	371	407	73	(23m)
Normals.....	87–123		205–274		306–376		Up to 150		

a = before splenectomy.
b = after splenectomy.
* Case of hemolytic jaundice.

On the whole, investigators seem now to agree that the total porphyrin excretion in both acquired and hemolytic jaundice is definitely increased. After splenectomy the output decreases both in urine and in feces, as shown by Dobriner (23a, m) and Watson (156f). Dobriner and co-

workers (23m) called attention to the association of the increased orderly type of hematopoietic activity in this disease with an increased formation of coproporphyrin type I.

Porphyrins in hemorrhage. Duesberg (25a) and others produced anemia in rabbits by hemorrhage as well as by distilled water and chemicals. The rate of porphyrin excretion in the urine was measured and no increase observed. More prolonged studies with determinations of fecal levels should have been made. Dobriner, and Dobriner and Rhoads, followed the urinary and fecal coproporphyrin levels in dogs after hemorrhage (23g) and with hemolysis from phenylhydrazin (23d). Coproporphyrin type I was excreted in both feces and urine in the control periods. Eight to twelve days after treatment, and when blood regeneration was well established, a marked increase in the rate of excretion of the same compound was obtained. In figure 5 the results of the experiment with phenylhydrazin are shown. A similar observation was reported by Dobriner et al. (23n) in a case of polycythemia in which phenylhydrazin was administered.

These experiments were made to demonstrate that type I and type III porphyrins are produced simultaneously as the products of the same synthesis.

Thiel (145a, b), Brugsch (15a, c), and Vannotti (152b), observed in some patients with hemorrhage into the intestine an increased rate of porphyrin excretion in the urine (143 to 175 micrograms daily), but in other cases found normal levels. Following venesection, phenylhydrazin administration, and thrombocytopenic purpura, normal urinary levels were observed. Langen and Grotepass (81a, b) followed the blood prophyrin levels (protoporphyrin) in animals after hemorrhage and chemical hemolysis. They found increased levels as regeneration proceeded.

Porphyrins in aplastic anemia. Brugsch (15a, c) in a patient with aplastic anemia observed low, and Vannotti (152b) in a similar case, increased urinary porphyrin levels. Dobriner, Rhoads and Hummel (23i), in six cases of aplastic anemia observed in four the excretion of coproporphyrin I and III in the urine and feces. In two more coproporphyrin III could not be identified by melting point determinations, but its presence was suggested by the chemical data. The patients' porphyrin excretion was followed for long periods, and the influence on it of vitamin A and vitamin C, as well as liver extract, was investigated. The data for the control periods is given in table 7. Because of the finding of type III coproporphyrin, Dobriner, Rhoads,

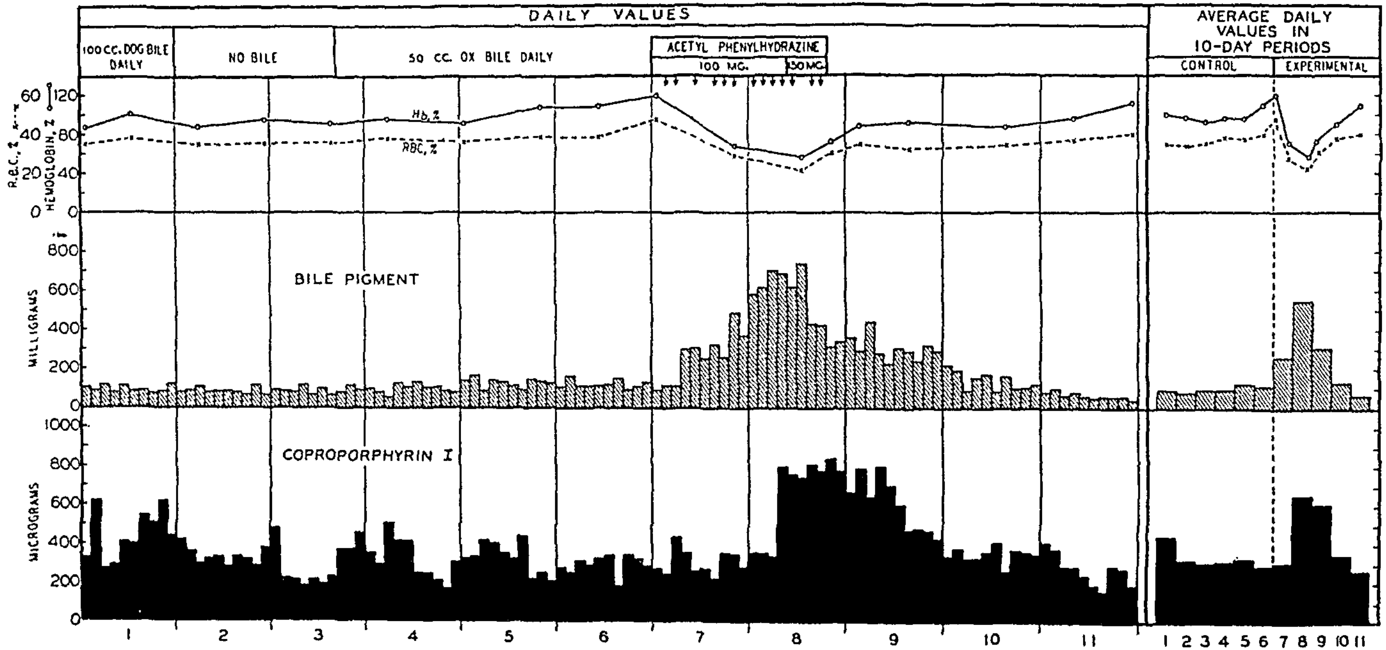


Fig. 5. A chart showing the rate of excretion of bile pigment and coproporphyrin I by a dog with hemolysis by phenylhydrazin. The high coproporphyrin values in the first period are due to the coproporphyrin present in the administered dog bile.

and Hummel suggest that the disease may be due to the action of toxic factors which result in the excretion of an abnormal porphyrin, as is the case in poisoning by lead and aromatic amines.

In sickle-cell anemia Dobriner, Rhoads, and Erf (23g) observed a marked increase of the total porphyrin excretion by a negro child. The average daily level was 470 micrograms for an 18-day observation period, and from the urine and feces coproporphyrin I was isolated and identified.

In erythroblastic anemia in a 4-year old child, Tropp and Penew (147b) observed daily urinary coproporphyrin levels of between 108

TABLE 7

The average daily total excretion of coproporphyrins by patients with aplastic anemia

The rate of excretion of coproporphyrin and urobilinogen, the blood levels, and the type of the bone marrow in the control period as well as the type of coproporphyrin excreted

CASE NUMBER	CONTROL PERIOD	AVERAGE DAILY COPROPORPHYRIN EXCRETION			AVERAGE DAILY UROBILINOGEN EXCRETION	RED BLOOD CELLS	HEMOGLOBIN	WHITE BLOOD CELLS	BONE MARROW	TYPE OF COPROPORPHYRIN EXCRETED
		Urine	Feces	Total						
	days	*micrograms*	*micrograms*	*micrograms*	*mgm.*	*millions*	*per cent*			
1. H. D.	9	89	122	210	83	1.100	21	1000	Hypoplastic	I (and III?)
2. C. S.	12	65	178	243	133	1.230	35	800	Hypoplastic	I and III
3. F. Y.	9	220	197	417	99	2.400	70	2250	Immature	I (and III?)
4. G. M.	12	212	280	492	370-450	1.110	22	950	Normally cellular	I and III
5. W. G.	9	550	428	978	273	1.42	27	1300	Hyperplastic	I and III
6. A. C.	21	201	493	694	128-165	1.440	39	2500	Hypoplastic	I and III
Normals	9	87-123	205-274	306-376	150					I

and 256 micrograms, and after splenectomy normal levels in the urine were found.

Leukemia. Duesberg (25a) and Thiel (145a, b) found no increase of coproporphyrin in the urine in myeloid leukemia. Brugsch (15a, c) reported normal values in one case, and in another a daily excretion of 186 micrograms. Lorente and Scholderer (90), as well as Vannotti (152b), observed an increased urinary porphyrin excretion (83 to 221 micrograms) daily. No studies of the porphyrin content of the feces in leukemia have been reported.

Hodgkin's Disease. In cases of Hodgkin's disease Duesberg (25a)

and Brugsch (15c) observed a normal rate of coproporphyrin excretion in the urine. Dobriner (23a, b) in severe febrile cases shortly before death observed daily excretion rates of 200 to 600 micrograms of coproporphyrin in the urine. Coproporphyrin I was identified from the urine of 5 patients and from the urine and feces of one.

Polycythemia. In a case of polycythemia rubra (Vaquez) with hypoplasia of the red bone marrow, Vannotti (152b) found a daily urinary porphyrin excretion of 273 to 398 micrograms, with a corresponding increase of the coproporphyrin in the plasma and of porphyrin (protoporphyrin?) in the red blood cells. Brugsch (15c) found 93 micrograms in the urine of one patient.

In a case of polycythemia vera Dobriner and co-workers (23m) found in the urine and in the feces, a total average excretion of 371 micrograms per day over a 6-day period; this value may be considered in the high normal range for the age of the patient. The porphyrin was identified as coproporphyrin I in both urine and feces. Günther (50a), Vannotti (152b), and Carrié (17a), found normal levels in the urine of similar patients, and Vannotti (152b), no increase of the plasma porphyrins. Carrié (17a) observed a patient under x-ray treatment whose urine showed an increase of coproporphyrin as anemia developed.

Deficiency diseases. Pellagra. The association of light sensitivity with increased porphyrin excretion has directed attention to the porphyrin metabolism in pellagra. Massa (99) saw increased porphyrinuria in endemic pellagrins in Italy; a finding which was confirmed by Bassi (6), by Ellinger and Dojmi (29), Beckh, Ellinger, and Spies (7). Spies and co-workers (137) studied patients with alcoholic and endemic pellagra and stated that they excreted increased amounts of porphyrin. The methods employed by Spies et al. are discussed by Dobriner and Rhoads (23k), and Watson (156g). Dobriner (23b) isolated coproporphyrin I from the urine and feces of a case of alcoholic pellagra in 1937, and the findings strongly suggested the presence of coproporphyrin III. Dobriner, Strain, and Localio (23n) reported studies of the urinary and fecal excretion in alcoholic pellagra before and after therapy. In the urine during the control period 254 mcg. coproporphyrin were excreted daily, and the total urinary and fecal output during the control period of 6 days was 897 micrograms with large amounts of protoporphyrin and deuteroporphyrin in the feces, whereas after treatment the total amounts of coproporphyrin dropped to normal. Spies and co-workers (137) mention the isolation of coproporphyrin III from the urine of 2 cases but chemical proof of its type was not presented. Wat-

son (156g) reports studies of the porphyrin excretion of 3 alcoholic pellagrins during a 10 day observation period and while under treatment with nicotinic acid. In all 3 he observed high levels in the urine (up to 425 micrograms) during the control period, and a sharp decrease after therapy. He isolated and identified coproporphyrin type III from one case.

The effect of coproporphyrin types I and III in sensitizing to light is discussed elsewhere; but the rôle of these substances in causing the dermatitis of pellagra is still unclear. In view of the heightened excretion of porphyrins in alcoholism and the excretion of coproporphyrin III in the intoxications associated with damage to the liver, a dysfunction of that organ in pellagra is suggested. It would be interesting to know precisely the facts concerning the porphyrin metabolism in cases of endemic pellagra without alcoholism. Brunsting, Brugsch, and O'Leary (16b) reported a normal urinary output in two cases of secondary pellagra.

Roncoroni (120) observed an increased porphyrin excretion by guinea pigs which had been fed a corn diet to produce experimental pellagra.

Sprue. Vannotti (152b) reported studies of three cases of sprue, in two of which the urinary coproporphyrin excretion was normal. In the third there was an increased output in the urine (100–170 micrograms). A large amount of a mixture of coproporphyrin and deuteroporphyrin was found in the feces. He administered riboflavin and liver extract and found that the rate of excretion decreased considerably.

Porphyrins in diseases of the skin. The association of increased rates of excretion of porphyrins of different types with disorders marked by light sensitivity has been referred to previously. A number of observations are recorded concerning various rates of urinary porphyrin excretion by patients with disorders of the skin. In many instances the methods employed are not stated, and because of this fact the results are difficult to evaluate.

Günther (50a) has reviewed the earlier literature. Goeckermann and co-workers (45) found increased urinary levels in the case of eczema, and Marquardt (97) reported similar findings in salvarsan dermatitis, eczema and other conditions. Hübner (67) reported levels as high as 600 micrograms daily of urinary porphyrins in 8 of 32 cases of erythema multiforme, and claims to have found uroporphyrin as well as coproporphyrin. His methods, however, are open to question. Scolari (131) found an increased excretion in erythroderma and other skin affections, and Ludy and Corson (91) an increase in lupus erythematosis,

an observation which could not be confirmed by Brunsting and his co-workers (16b). The writers had an opportunity recently to examine 24-hour collections of urine from cases of this disorder; in three no increase of porphyrin content was observed, while in one a high level was found shortly before death. McFarland and Strain (104), and Tropp (147c), recently reported studies of a series of cases of skin disease. In a case of xeroderma pigmentosa unequivocally high levels were found. Zoon (162) saw in the same condition a high urinary and high blood porphyrin levels. Brunsting and co-workers (16b) reported studies of the urinary porphyrin excretion in a large series of cases of diseases of the skin, and in no instance was uroporphyrin found. High rates of urinary excretion were found in cases of exfoliative dermatitis and in extensive bacterial ulcerations of the skin, in widespread burns, erythroderma, Hodgkin's disease of skin, erythema multiforme and pemphigus vulgaris. Of nine cases of eczema solare and urticaria solare, only two were found to have a slight increase of porphyrin output. In one case of pityriasis rosea the fecal porphyrin excretion was high and the urinary output normal. The only other high fecal values were observed in cases of salvarsan dermatitis.

Porphyrins in mental diseases. Very few reports are available concerning the porphyrin excretion in mental disturbances. Brugsch (15e) did not find an increase of the porphyrin output in the urine, but in two cases of excited schizophrenic patients he found levels of 181 and 184 micrograms. Scheid and Libowitsky (84) reported an increased coproporphyrin I production and excretion during the febrile attacks in schizophrenic patients.

Porphyrins in toxic states. Lead. One of the earliest examples of toxic porphyrinuria was lead poisoning, described by Garrod (41b) in 1892, and by Stockvis (139) in 1895. The literature up to 1925 has been discussed in detail by Günther (50a, b) and needs no further consideration.

All agree that coproporphyrin is present in increased amounts in the urine of patients with lead poisoning. Grotepass (48a, d) identified the pigment as coproporphyrin type III, an important observation since verified by Watson (156e), Vigliani and Waldenström (153c), and Mertens (105a). Dobriner (23s) isolated and identified a large amount of the type III compound and a small amount of type I in the urine of several patients with acute symptoms. From the feces Watson (156f), and Vigliani and Libowitzky (153d), isolated coproporphyrin Type I. Dobriner (23g) however, obtained a small amount of copro-

porphyrin type III as well as coproporphyrin type I. Vigliani and co-workers (153g), Watson, Dobriner and Mertens, found protoporphyrin in the feces. Both feces and urine have been studied in a few instances. Watson (156f), as well as Vigliani and Libowitzky (153d), found coproporphyrin III in the urine and coproporphyrin I in the feces. Dobriner (23b, r) obtained large amounts of coproporphyrin III and small amounts of type I from the urine. In the feces, however, the order was reversed.

The porphyrin content of plasma and erythrocytes in lead poisoning was studied by Vigliani and Angeleri (153b, g), who found increased amounts of protoporphyrin and in certain instances traces of coproporphyrin. Günther (50a) reviewed the quantitative studies. The patients of Grotepass (48d), Schreus and Carrie (129c), Vigliani with Sasso et al. (153a, g), Vigliani and Libowitzky (153d), Roth (121a), Vigliani and Waldenström (153c), Frank and Litzner (38c), Vannotti (152b), and Mertens (105a) excreted between 630 and 14,800 micrograms daily in the urine.

Very few quantitative determinations of coproporphyrin in the feces of patients with lead poisoning have been made with adequate methods. Mertens (105a) studied one case and obtained normal values. Dobriner and Strain (23r) observed several cases during and after attacks of acute colic. In both periods increased amounts of coproporphyrin and protoporphyrin, as well as small amounts of deuteroporphyrin, were excreted.

Increased light sensitivity has been reported by Carrié (17a), Roth (121a), and Vannotti (152b). Hijmans v.d.Bergh and co-workers (61b) administered lead salts to human beings, and observed increased urinary porphyrin excretion after from 2 to 12 days. Vigliani et al. (153a, g) in similar studies found the urinary porphyrin excretion in the control period averaged 51 micrograms per day; on the fourth day of poisoning it rose to 91 micrograms, and at the end of 9 days had reached 522 micrograms per day. After treatment was discontinued the excretion decreased slowly and reached normal levels after four weeks. No determinations of coproporphyrin levels in the feces were made. It has been suggested by some investigators that the increased porphyrin excretion which marks acute lead poisoning may be of diagnostic value (Günther, 50a; Franke and Litzner, 38c). Roth (121a) did not see any effect of liver therapy on the porphyrin excretion, but Schreus and Carrié (129c) thought that they influenced it by Campolon injections.

In 1895 Stockvis (139) produced by lead poisoning in rabbits an increased excretion of urinary porphyrins, and the experiments have been repeatedly confirmed. The earlier literature is reviewed in the publications of Günther (50a, b), Liebig (86), and Duesberg (25a). Fischer and Duesberg (35m) isolated coproporphyrin III from the urine of rabbits with lead poisoning. No increase of coproporphyrin was found in the feces. Waldenström (155a) confirmed the findings regarding coproporphyrin type III, but isolated coproporphyrin I from the urine of one animal. Uroporphyrin could not be detected, but an exceedingly small amount of an ether-insoluble, but ethyl acetate-soluble, porphyrin (uroporphyrin III?) was found. In the bile Waldenström found coproporphyrin and protoporphyrin.

The bone marrow in experimental lead poisoning was studied by Liebig (86), Duesberg (25a), Emminger and Battestini (30a), and Vannotti (152b). Liebig extracted relatively large amounts of porphyrin of an unknown type and Waldenström (155a) obtained copro porphyrin. The latter states that free protoporphyrin was not present. Emminger and Battestini (30a) obtained evidence that by the administration intravenously of calcium chloride to animals poisoned with lead, the increased excretion of porphyrin in the urine could be restored to normal and the bones were no longer fluorescent. If lead and calcium chloride were given simultaneously no porphyrinuria developed, nor could evidence of increased erythropoiesis in the marrow be obtained. It is unfortunate that no determinations of fecal porphyrins were made in this study.

Sulfonal. Porphyrinuria in sulfonal poisoning was reviewed by Günther (50a, b), who reported 66 cases, of which 92 per cent were female. The same author reported 11 patients with trional poisoning, of which 9 were females. The symptoms are in many respects similar to those of acute porphyria. Ellinger and Riesser (27) isolated from the urine of a patient with trional poisoning a uroporphyrin melting between 255°C and 257°C, a finding which suggested that the compound was of type III. Dobriner (23a) from a case of sulfonal poisoning isolated a uroporphyrin melting at 280°C, an indication that it was type I with a small amount of type III. Germuth (43) and Brugsch (15a, l) proved that sulfonal and trional increased the excretion of porphyrins by patients, and the former felt that the substitution of the ethyl radical for the methyl group increased the porphyrin production.

Stockvis (139) produced an increased excretion of porphyrin in the urine by feeding sulfonal to rabbits and to dogs. A thorough investi-

gation of the subject was made by Neubauer (112). Duesberg (25a) could induce porphyrinuria in rabbits, as could Laubendender and Monden (82) and Polson (115). Fischer and Zerweck were not able to do so, however. Fischer and Duesberg (35m) found uroporphyrin, but no coproporphyrin in the urine of rabbits with sulfonal poisoning, and from the feces of the animals a porphyrin with a melting point of 188°C could be isolated. The same compound was isolated in lead poisoning, and in much smaller amounts, from normal rabbit feces. Waldenström and Wendt (155g) poisoned rabbits with dimethylsulfondimethylmethan and caused a temporary, inconstant, increase of urinary porphyrin. This was identified by Waldenström as coproporphyrin type III. In two animals a suggestion of an ether-insoluble porphyrin (uroporphyrin?) was found.

Salvarsan. Cavina (18), Schreus (129a), Carrié (17a), and Scolari (131) investigated patients receiving salvarsan and found an increase in amount of coproporphyrin in the urine. Hoerburger and Fink (64b) identified it as a type III compound. Marquardt (97), Brugsch and O'Leary (16b), observed increased levels of coproporphyrin in the urine in some cases of salvarsan dermatitis (199 to 684 micrograms daily). The latter authors reported a fecal excretion of 1328 micrograms of coproporphyrin daily in one case, but in three others no increase was present.

Alcohol. Franke and Fikentscher (38b) and Franke (38a) investigated the influence of the administration of alcohol to human beings on the excretion of porphyrin in the urine. They found that moderate amounts increased the output to double the normal level. Brugsch (15e) and Brugsch and Keys (15g) found that in chronic alcoholism the porphyrin output in the urine was greatly increased (500 to 2,000 micrograms daily), whereas the fecal excretion was reported to be low. Coproporphyrin type III was isolated in one instance, and a small amount of coproporphyrin type I was present also. From another case only the type I compound was isolated. The excretion of type III compound in alcoholism should be kept in mind in view of the similar finding in alcoholic pellagra, and in certain cases of liver disease.

Phosphorus. In phosphorus poisoning in animals a great increase of urinary porphyrin excretion was observed by Lorente and Scholderer (90), Perutz (113), and Thomas (146b).

Selenium. Halter (52) published a report of a patient with selenium poisoning who showed an increased excretion of porphyrin in the urine. Vannotti (152b, l) reported a case of mercury poisoning with the ex-

cretion of uroporphyrin and coproporphyrin type I in the urine. The coproporphyrin output was 4,000 to 12,000 micrograms daily. The patient showed signs of light sensitivity and after two years still excreted 200 to 1300 micrograms of coproporphyrin.

Dibenzanthracene. Dobriner and Rhoads (23j) observed increased urinary porphyrin levels in rabbits treated with 1,2,5,6,dibenzanthracene. Coproporphyrin types I and III were excreted in nearly equal amounts. Normal rabbits excrete small amounts of coproporphyrin III.

Barbiturates. The influence of barbiturates on the excretion of porphyrins was reviewed by Günther (50a, b) but he reports no clear-cut results. Brugsch (15e) studied the same subject in psychopathic patients and observed a doubtful increase in rare instances. Miscellaneous observations of increased porphyrin excretion in poisoning by sedatives are reported by Haxthausen (58), Duesberg (25c), and Fischer and Duesberg (35m). The latter isolated uroporphyrin I in a case of intoxication of unknown nature. Laubender and Monden (82), in an experimental study found no increase in the urinary porphyrin excretion by rabbits fed barbiturates.

Sulfonamides. Brunsting (16a) and Brunsting and co-workers (16b) studied several patients with dermatitis and photosensitivity resulting from treatment with sulfanilamide, and in two found increased amounts of porphyrin in the urine. In a detailed study Rimington and Hemmings (118g) observed rates of coproporphyrin excretion daily in the urine up to 463 micrograms. They established the fact that coproporphyrin types I and III were excreted in equal amounts. The feces were not investigated. Silver and Elliot (134) demonstrated an increased amount of porphyrin in the urine of patients receiving sulfanilamide, but McFarland and Strain (104) did not find any increase.

Rimington and Hemmings (118g) reported an increased excretion of coproporphyrin in the urine and feces of rats treated with sulfanilamide. They identified coproporphyrin III and obtained suggestive evidence of the presence of traces of type I in the urine. In the feces a mixture of types I and III was present. The treated animals excreted 2½ to 10 times as much porphyrin as normals. The protoporphyrin present was identified as a type III compound and careful investigation revealed no type I. Wien (159) repeated and confirmed Rimington's experiments, but found no porphyrinuria after sulfapyridine administration.

Rimington and Hemmings (118f, h) reported extensive animal experiments concerning the porphyrinuric action of 27 compounds re-

lated to sulfanilamide and other aromatic amines. They observed an increased excretion of coproporphyrin III in those cases which showed methemoglobin formation in the blood. Brownlee (14) isolated coproporphyrin III and small amounts of coproporphyrin I from the urine of rats treated with acetanilid, phenacetin, phenazone, amidopyrine, aspirin and p-aminophenol. None of the authors mentioned describes the type of porphyrin excreted by normal rats. Since Dobriner and Rhoads (23j) observed the excretion of coproporphyrin III in the urine of normal rabbits it seems to be of the utmost importance to know the type of coproporphyrin excreted by normal rats. In illuminating gas intoxication Tropp and Penew (147b) found a large amount of coproporphyrin in the urine. The type was not identified.

Coproporphyrin type III theoretically can be produced by abnormal breakdown or synthesis of type III porphyrins contained in the respiratory pigments. It is produced in cases of intoxication by lead and other substances, as well as in certain diseases. Hijmans v.d. Bergh (61b) has shown that coproporphyrin is formed by the liver from protoporphyrin. Recently Rimington and Hemmings (118f, h) and independently Brownlee (14) advanced the idea that coproporphyrin type III may be produced after the formation of methemoglobin, "which, when once formed, is degraded, in part at least, by a mechanism which leads ultimately not to bile pigment but to porphyrins. Hematin may possibly represent one of the intermediate stages in this transformation" (Rimington); or, as Brownlee says, "where hemoglobin is oxidized to methemoglobin, the normal conversion into bilirubin cannot occur but is replaced by degradation to coproporphyrin III."

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THE MECHANISM OF ACQUIRED IMMUNITY IN INFECTIONS WITH PARASITIC WORMS

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The early immunological studies on helminths were for the most part limited to various immunological and serological tests that could be used in diagnosis. These investigations yielded many interesting facts regarding immunological relationships of the worms and several practical diagnostic aids as, for example, those used in hydatid disease. Furthermore, from the standpoint of the mechanism of acquired immunity, they demonstrated that specific antibodies and specific hypersensitive states are the rule rather than the exception in worm infections. During this time most immunologists and parasitologists considered that the larger animal parasites would not stimulate any demonstrable acquired immunity which would inhibit the development of or kill the parasites. In general, recovery and elimination of infection were presumed to be largely an expression of old age and death of the parasites. About 10 years ago, however, investigators began to collect evidence that infection with some of the metazoan parasites prevents superinfection, and recovery from infection results in a greater or less acquired immunity to subsequent infection. Such cases are now being reported with increasing frequency.

In the present review, emphasis will be placed on those few helminths which have been studied from the standpoint of the mechanism of acquired immunity. Specifically, I shall consider in detail the work on certain larval tapeworm infections, where the antibody basis for immunity has been furthest analyzed, and on *Nippostrongylus muris* of the rat, where the mechanism of acquired immunity has probably been studied from the most angles. I shall then attempt to correlate these data with the findings on the other metazoan infections. Studies on the actual demonstration of acquired immunity will be omitted

except for the few forms considered in this review. The extremely interesting series of investigations on natural and age (which includes both natural and acquired factors) immunity and on immunologically nonspecific factors, such as diet, size of infection and age and genetic constitution—all of which affect both natural immunity and the ability to develop acquired immunity—will only be considered from a general point of view. All other immunological and serological data will be omitted from discussion.

The earlier immunological work on parasitic worms is surveyed by Taliaferro (1929) and the more recent work by Culbertson (1938). Comprehensive reviews, according to Culbertson, are given by di Aichelburg (1936) in Italian and by Schulz and Shikhobalova (1935, 1937) in Russian. Special aspects of the subject are treated by Cameron (1934 and 1937), Chandler (1932b), Clapham (1933), Kotlan (1934), Peters (1936), Sandground (1929), Schmid (1936) and Taliaferro (1934).

I. THE UTILITY OF PARASITIC WORMS FOR IMMUNOLOGICAL STUDIES. All evidence indicates that the humoral and cellular mechanisms operative in acquired immunity against helminths are fundamentally identical with those operative against other infectious and noninfectious antigenic agents (see Taliaferro, 1934). Accordingly, the nature of the helminths and the type of infection they produce give them certain peculiar advantages and disadvantages as material for immunological studies on acquired immunity to infection. The chief points of interest are as follows:

Inasmuch as most worms do not reproduce in the body, each adult worm represents one infective larva. This permits the quantitative control of the infective dose and resulting infection in a far more exact manner than can be attained with bacteria or protozoa which multiply and accumulate in the host at varying rates.

Most of their outstanding advantages are related to their large size. Thus, because their tissues can be isolated, immunological studies can be made on tissue specificity, as was done by Canning (1929) in *Ascaris*. The larger forms furnish abundant material for immunization, for the preparation of the antigens, for the isolation of specific carbohydrates and for other chemical studies (cf. the large quantities of immunologically active polysaccharide prepared by Campbell, 1936a, from *Ascaris*). In addition, their course in the body can be followed and the effects of immune processes on them can be ascertained in a way that is impossible with most bacteria. An intermediate position in this last

respect is held by the protozoa which have been studied extensively from the standpoint of the actual effect of immune processes.

The helminth parasites have, on the other hand, certain specific limitations as material for studying immunity, chief among which is that no investigator has so far succeeded in cultivating them for long periods outside of the host. The fact, however, that Glaser and Stoll (1938) reported the rearing of the larvae of *Haemonchus* under sterile conditions outside of the body to a point in the fourth larval (second parasitic) stage seems to indicate that these difficulties may to some extent be eventually overcome. Furthermore, results, such as reported by Sarles (1938), demonstrate that worms can be kept in serum outside of the body long enough to study the effects of antibodies on them.

II. Methods of demonstrating immunity. Immunity may be complete—at least in the sense that no developing or mature forms can be found. Frequently, however, infective stages penetrate the first barriers of the immune animal, but, in comparison with the normal host, fewer worms start development, more are delayed in migrating and are arrested in their development, and some may die. In the case of intestinal infections, those that do develop may be stunted and eliminated more quickly from the body. Stunting is frequently associated with the production of fewer eggs by the females (lower fecundity or inhibition of reproduction).

Determination of the absolute number of worms (worm burden) is generally obtained after killing the host. In most intestinal infections and in somatic infections with large parasites, such as the larval tapeworms, the worms are generally counted directly. In other cases, and particularly in somatic nematode infections, the worms can be separated and concentrated after macerating and digesting the host tissue in an artificial gastric juice of pepsin and hydrochloric acid. Such a method gives no information about dead parasites because the latter are digested along with the host tissue. Motile worms which react positively to heat can, after digestion of the host tissue, be concentrated in the Baermann apparatus which was originally devised for the isolation of hookworm larvae from the soil. Nonmotile worms can be concentrated by washing and sedimentation. Occasionally, motile worms can be concentrated from macerated tissue in a Baermann apparatus without preliminary digestion. A few intestinal parasites can be effectively removed by anthelminthics without sacrificing the host. Once obtained, the parasites can be counted and their relative development ascertained.

In infections with intestinal parasites or where eggs are passed in the feces, the degree of immunity can be ascertained approximately by using Stoll's egg-count method (or a modification of it) which has the advantage of not necessitating killing the host. This method consists essentially in determining the number of eggs passed per gram of feces or per day and, in conjunction with the average number of eggs produced per female and the average sex ratio, gives a fair approximation of the number of worms in the intestine. It was devised for work under field conditions where the infections generally consist of worms of different ages which have been subjected to varying immunological factors. In the study of single infections and particularly of infections in immune animals, the results obtained must be interpreted with due regard to such facts as the following: 1, the number of eggs produced per female (fecundity) varies with the age of the female and, hence, with the length of infection (see for example Herrick, 1928, and Sarles, 1929a); 2, the fecundity of females is generally lower in heavy infections (see for example Hill, 1926, for hookworm of man; Sarles, 1929a, for hookworm of dogs; Winfield, 1933, for *Heterakis* in rats); 3, the sex ratio may change, especially during the latter part of the infection; and finally, 4, females under the influence of immune processes often produce fewer eggs (see work on *Nippostrongylus*).

In addition to the preceding measures of resistance, which are expressed as effects on the parasite, pathological effects on the host have also been used. Such measures vary all the way from estimations of general malaise through specific pathological effects (for example, the number of local hemorrhages in the lungs of rats infected with *Nippostrongylus* as ascertained by Sarles and Taliaferro, 1936) to determinations of the death of the host. Nevertheless, the fact that an immune host exhibits comparatively minor discomfort from an infection whereas a control exhibits pronounced ill effects from an infection of equal degree does not necessarily indicate specific antitoxic effects, as suggested by certain authors. It may simply indicate that the parasites in the immune host are stunted and their general activity retarded.

III. Natural immunity and various nonspecific factors influencing infection. In evaluating acquired immunity it is essential to eliminate, as far as possible, natural immunity which is independent of past infection or immunizing procedures and which rests predominantly on nonspecific factors in the immunological sense. Where immunity is measured solely in terms of resistance to invasion, the immunity can be definitely classified. In most cases, however, it is measured in terms of effects on the parasite which has had an appreciable

sojourn in the host and which, therefore, may have encountered factors of natural immunity at first and those of acquired immunity later. This condition is exemplified in initial infections with *Nippostrongylus* where, in addition to natural immunity, acquired immunity develops during the second week and results in the expulsion of most of the parasites from the intestine at about the end of the second week.

In spite of the difficulty of separating natural from acquired immunity, a few generalities regarding natural immunity and immunologically nonspecific factors may be pointed out. On the one hand, absolute natural immunity is found against the helminth parasites as against other invading organisms and is indicated by the limitation of certain parasites to specific hosts. The worms differ from the protozoa, however, in that they more frequently develop in abnormal hosts, so-called accidental parasitism. In such hosts they may pursue their course for varying periods. They may complete their usual development, may survive for only a short time or may be arrested at various stages of their development. On the other hand, even the most susceptible host may be unsuitable as a medium for the development of all of the parasites. Data on this point are interesting in spite of the fact that such matters as probable damage to the larvae before infection are difficult to evaluate. Thus, to cite only two examples, Shorb (1933) found that less than one per cent of *Hymenolepis fraterna* develop in rats and mice, and Scott (1929) found that only 45 per cent of a cat-adapted strain of *Ancylostoma caninum* develop in young kittens. These data may be similar to the findings in malaria where only a small percentage of the forms produced by reproduction survive in the nonimmune host under the most favorable conditions (see review by Taliaferro and Mulligan, 1937).

The literature on immunity to infection with helminth parasites has to a large extent been concerned with natural immunity, age immunity and various immunologically nonspecific factors which influence the development of the parasites (see review by Culbertson, 1938). From the standpoint of the present review, this work shows clearly that the following factors have to be controlled in experiments on acquired immunity either because they affect natural immunity or because they introduce nonspecific factors which affect the development of the parasite, the development of natural immunity or the acquisition of acquired immunity:

1. Natural immunity to a given parasite obviously varies with the species of host, but also with the genetic constitution of individuals of the same species of host.

2. Age immunity develops with age. It undoubtedly consists of natural immunity and probably of the ability to develop acquired immunity.

3. Diet, including experimental avitaminosis, affects both natural immunity and the ability to develop acquired immunity.

4. The fact that the degree of infection influences the fecundity of the females has already been mentioned. The same is true for the development of the parasite. As a rule, as the infection becomes heavier, the survival, the rate of development and the final size of the worms decrease.

5. In at least one infection, the larval form of *Taenia taeniaeformis* of the rat, the development of acquired immunity is associated with sex and can be apparently modified by treatment with sex hormones (Campbell, 1939b).

IV. Acquired immunity. A. *Mechanisms of Host Resistance.* The life cycle of each form considered in this review is briefly described because it is frequently complex and determines the type of infection, the intimacy of contact with the tissues, the location of the points of defense and probably the degree of immunity.

1. *Taenia.* Most of the cestodes which have been used in studying immunity belong to the family Taeniidae, and typically show an alternation of hosts. In the species considered in this review both hosts are mammals. The adult tapeworm occurs in the lumen of the intestine of the definitive host with its scolex or "head" more or less intimately attached to or buried in the mucosa. The larval tapeworm (cysticercus, hydatid, etc.) inhabits various parenteral tissues of the intermediate host where it may migrate extensively.

The adult tapeworm, *Taenia taeniaeformis* (= *T. crassicollis*), is a parasite in the intestine of the cat. Its eggs, when swallowed by rats or mice, liberate larvae (onchospheres) which penetrate the intestinal mucosa and are carried by the portal blood system to the liver where they develop into bladder-like forms which are often designated *Cysticercus fasciolaris.* The scolex becomes evaginated from the bladder and may develop a chain of segments (strobilocercus).

a. *Humoral phases of immunity.* Although an acquired immunity against this infection was postulated as early as 1888 by Vogel because he frequently found only one cysticercus in wild rats or mice, it was first demonstrated by Miller (1931b). Miller found that animals with a few or many cysts of the larval form of *T. taeniaeformis* in the liver are protected against large numbers of onchospheres fed from 56 to 155

days thereafter and are more effectively protected than artificially immunized animals. Furthermore, removal of the cysts from the liver does not appear to decrease the immunity within a period of 60 days (Miller and Massie, 1932). Artificial, active, acquired immunity after repeated injections of freshly ground or powdered adult tapeworms is striking within a period of 167 days, as evidenced by the almost complete *inhibition of the growth of the cysticerci in most rats* (Miller, 1931a and 1932b). Fresh material retains its potency for at least 3 months when frozen and kept in evacuated ampoules, and powdered material is similarly active after the lipoids are removed. The injection of an efficient antigen after the rats are infected does not cause inhibition of cyst development (Miller, 1932b). In studying the antigenicity of nonspecific worm materials, Miller (1935a) found that partial protection against larval infection with *T. taeniaeformis* can be induced in rats by feeding the larval forms of *T. pisiformis* or by introducing the adult form of the same species intraperitoneally, whereas no protection against the same form is afforded by dried materials of *T. pisiformis* and several other cestodes (see also Miller, 1932b).

An antibody basis for this immunity was demonstrated by Miller and Gardiner (1932) who showed that serum from rats actively immunized by infection is effective in inhibiting cyst development in normal rats in doses sometimes as low as 0.25 cc. per 100 gram rat. Passive immunity with serum lasted for 26 days and in 2 of 4 rats for 36 days. Serum from *artificially immunized rats is less active than from infected* animals and serum from rabbits similarly immunized is only slightly protective. Miller (1934) ascertained that immune serum is curative, i.e., arrests the development of cysticerci already growing in normal rats. An appreciable degree of immunity is passively transferred to offspring from infected mothers and lesser degrees from artificially immunized mothers (Miller, 1935b). Various additional facts were brought out by Miller and Gardiner (1934). Of particular interest is the fact that Miller (1932a) found that kittens and cats infected with adult tapeworms of *T. taeniaeformis* are not protected against superinfection with additional intestinal forms.

Taenia pisiformis is one of the commonest tapeworms of dogs and cats, and the larval form, often designated *Cysticercus pisiformis*, develops in the liver and mesentery of rabbits.

Miller and Kerr (1932) artificially immunized rabbits to varying degrees to the larval infection of this form by injecting ground fresh or dried adult worms. Kerr (1935) extended this work and, in addition,

found that infection in the rabbit results in immunity to superinfection, and that immunity can be passively transferred by doses of at least 2 cc. per 100 gram rabbit.

Findings of great theoretical importance on the mechanism of antibody-action against larval tapeworms have been brought out in a series of papers by Campbell. He (1936b) demonstrated that the total immunity of rats to the larval stage of *T. taeniaeformis* can be divided into an "early" acquired immunity, which prevents larvae from developing to a recognizable stage in the liver, and a "late" acquired immunity, which results in the death of the cysticerci after they have formed recognizable cysts. There is also a certain degree of "late" immunity in controls which represents a natural immunity. The two types of acquired immunity probably arise from the stimulation of different antigens in the worm because the relative amounts of the two types of immunity vary markedly following immunization with different fractions of the adult worms. Further study (1938a, b, c) indicated that the antibodies associated with "early" immunity arise within 1 week after infection, are produced by immunization with ground fresh larval worm material and can be absorbed from immune serum with such worm material; whereas the antibodies associated with "late" immunity arise several weeks after infection, are not produced by immunization with ground fresh larval worm material and cannot be absorbed from immune serum with such worm material. Campbell suggested that the antibodies associated with "late" immunity do not arise from artificial immunization and are apparently nonabsorbable because the antigens are elaborated by the living parasite and occur only in insufficient quantities in ground worm material. Another possibility may be that the antigens disappear rapidly from the parasite after death and grinding. Immune antienzymes and the nonabsorbability of antibodies are considered further under *Nippostrongylus*.

2. *Echinococcus.* The adult *Echinococcus granulosus* is a small tapeworm from the small intestine of the dog, wolf, jackal and fox. The larval stage or hydatid is found in sheep, cattle, pigs, camels and man.

The investigations on *Taenia* have dealt mainly with larval infections. It is, therefore, interesting that Turner, Berberian and Dennis (1933, 1936) produced a high grade but partial protection in the dog to the intestinal infection with *E. granulosus* by injecting phenolized or formalized suspensions of dried scolices and germinal membranes of fertile hydatid cysts from cattle. Using essentially the same antigen in an attempt to immunize lambs against the somatic larval infection

with the same parasite, Turner, Dennis and Berberian (1937) found that the immunized animals become infected, but with fewer cysts, and that the cysts show thicker surrounding adventitia, more calcification and degeneration of the germinal layer. The increased tissue response of these immunized animals should be compared with the results reported later for *Nippostrongylus*.

3. *Nippostrongylus*. The life cycle of *Nippostrongylus muris* is representative of a number of intestinal nematodes. The eggs are evacuated with the feces from infected rats and develop into infective larvae. The infective larvae, if they penetrate the skin of a rat, reach the lumen of the intestine via the skin, blood, lungs, trachea and esophagus. The adult worms are bisexual, measure 4 to 6 mm. in length and are generally found in the upper part of the small intestine. The development outside of the body takes about a week or less, migration through the skin and lungs and establishment in the intestines is accomplished in 2 to 3 days, females are mature and begin tó lay eggs 6 to 7 days after infection, and most of the adult worms are expelled from the intestine due to acquired immunity approximately 2 weeks after infection. During the first two weeks of the infection when the larvae are actively feeding in the tissues, with the coincident passage of secretions and excretions from their mouth, anus and excretory pore, and are growing and migrating through the skin, lungs and intestine, inflammation and cellular changes are minor and quickly disappear. Thereafter, the rat is immune and inflammation is more pronounced, as will be discussed hereafter.

In addition to age immunity, Africa (1931) and Schwartz, Alicata and Lucker (1931) reported that rats after recovery from infection with *N. muris* are relatively resistant to subsequent reinfection. These results have been verified and studied quantitatively from many angles by Chandler (1932a, 1935a, b; 1936a, b; 1937a, b; 1938), Spindler (1933, and 1936), Graham (1934), Porter (1935a, b, c), Sarles (1938, 1939), Sarles and Taliaferro (1936) and Taliaferro and Sarles (1939). A review and theoretical discussion are given in Chandler (1937b). Acquired immunity has now been produced not only by infection, but also by implanting the worms directly into the duodenum (Spindler, 1936; Chandler, 1936b). Various degrees of immunity can also be induced by the injection of dead worm material (Chandler 1932a, 1936b), by the death and disintegration of adults placed in the body cavity (Chandler, 1936b), and by the inoculation of living larvae of the closely related *Longistriata adunca* (Chandler, 1932a)—but not by the inocula-

tion of the less closely related larvae of *Ancylostoma caninum* (Chandler, 1937a).

The worms, according to Sarles and Taliaferro (1936) and Taliaferro and Sarles (1939), pursue the same course in immune rats through the skin, lungs and intestine as in normal rats, but the activities of the worms and the reactions of the host may be profoundly altered. In sufficiently immune rats, for example, larvae of a reinfecting dose are immobilized, stunted and delayed in their migration in the skin and lungs and a few may be killed, whereas those that eventually reach the intestines lay few eggs, continue to be stunted and are quickly eliminated via the intestinal tract. Many of the worms seem to be more or less engorged with masses of precipitate, and masses of similar appearing material may be found near them in the tissues. In addition, exudate cells rapidly mobilize and continue to be present for weeks or months in areas containing worms. In the skin and lungs, but not in the intestine, such cellular accumulations quickly form nodules around sufficiently immobilized worms and, if the worms die, the nodular macrophages eventually clear up the resulting debris.

Several investigators, and especially Chandler (1932a, 1937b), have emphasized that the immunity is evidenced largely by an inhibition of egg-laying and a retardation of growth and development, without in most instances producing lethal effects on the worms. These inhibitory effects are mainly temporary because Chandler (1936a) was able to show that stunted worms, transplanted from the intestines of immune to nonimmune animals, grow and start egg production. In highly immune animals, however, many worms do die in the skin and lungs.

a. *Humoral phases of immunity.* The present evidence indicates that humoral factors assume a predominant role in immunity. Chandler (1932a) first stressed the general nature of immunity to this parasite. Then, because of his inability to find evidence of an antibody by passive transfer (1935a), he stressed its local nature in the intestine and suggested a nutritional basis due probably to antienzymes. Later (1936b, 1937b, 1939) he divided the immune mechanism into "parenteral" and intestinal immunity. According to him, "parenteral immunity" is always general; arises from the antigenic stimulation incident upon the tissue migration of the larvae; and is associated with passively transferable antibodies, a heightened tissue response, retention and destruction of larvae in the parenteral tissues, a more rapid disintegration of adult worms transplanted to the peritoneal cavity and

a stunting of growth and inhibition of reproduction of the worms; whereas intestinal immunity, although it admittedly can be a part of the parenteral immunity, can be purely local and not transmitted to the blood. He maintains (1939) that the local character of the intestinal immunity is shown by its failure 1, to produce a heightened skin reaction to invading worms; 2, to influence the parenteral phase of development; 3, to be transferred to a parabiotic twin, and 4, to be passively transferred in serum.

Undoubtedly many manifestations of immunity and, particularly, those in the intestine may remain localized, but until more conclusive evidence is brought forward, the reviewer considers that the same humoral and cellular mechanisms, chief among which are specific antibodies, are involved in both Chandler's "parenteral" or general immunity, and local intestinal immunity. The observations so far accumulated can be explained on the basis that there is both a local production and a local recruitment of antibodies and cells. Antibodies, for example, are probably initially produced locally and become generalized only if their location, concentration and amount permit them to reach detectable concentration in the blood or if the antigens become generalized. On the other hand, once they reach appreciable concentrations in the blood, they may diffuse into local areas of defense because of various changes in capillary permeability associated with inflammation (Menkin, 1938).

According to Taliaferro and Sarles (1939), the mechanism of immunity in the skin, lungs and intestine (omitting from consideration the ultimate origin of antibodies from cells) is primarily humoral with secondary cellular coöperation. The same authors have pointed out that during the intestinal phase of the infection the worms pierce the epithelium and ingest antibodies (as evidenced by precipitates) and formed elements of the tissue and blood and pour antigenic secretions into the lamina propria. Actually, therefore, the worm in the intestine has an intimate contact with a lymphoid tissue and the blood and lymph.

1. Passive transfer of immunity. Passive transfer of immunity was first demonstrated by Sarles and Taliaferro (1936) and their findings have been corroborated and extended by Chandler (1938) and Sarles (1939). With the possible exception of the death of the worms in the skin, all the manifestations of immunity can be duplicated in normal animals by the passive transfer of serum from immune animals. Sarles (1939) has even found that immune serum given after the worms have become established in the intestine may result in their expulsion. The

antibody effects of precipitate formation, and the immobilization of the larvae and the prevention of their feeding, but, obviously, not the cellular phases, have been duplicated to various extents with immune serum *in vitro* by Sarles (1938).

2. The rôle of precipitins. The *in vitro* studies of Sarles (1938) and the *in vivo* studies of Taliaferro and Sarles (1939) indicate that the cuticle of the living worm is impervious to antigenic materials. The materials in the gut and those passed out of the mouth, anus and excretory pore are the chief inflammatory stimuli and act as precipitinogens. In the immune animal the precipitins formed against these excretions and secretions account for the precipitates formed in and around the worms and also undoubtedly induce the heightened allergic response in a way similar to, but milder than the Arthus phenomenon, described by Opie (1923, 1924a, b, c, d). Visible precipitates formed from these antigens are the center of inflammatory responses, but probably, as believed by Opie, even greater inflammatory responses result from the union of antigen and antibody within the host's cells without the formation of visible precipitate.

3. Other antibody effects. By analogy with the localization of foreign protein, it would seem entirely possible that the precipitins immobilize the parasite. In any case, the *in vitro* and *in vivo* experiments indicate that immobilization is primarily effected by humoral rather than cellular mechanisms. This fact is in accord with recent work by Hanger (1930), Rich and McKee (1932), Rich (1933) and Cannon and Hartley (1938) on the localization of highly virulent bacteria and is contrary to the ideas of Menkin (see review, 1938) and others that, although antibodies may later play an important part, the primary fixation is due to mechanical factors of inflammation, such as coagulation of plasma in edematous tissue, the occlusion of lymphatics by thrombi, etc.

The question then arises as to whether the worms are also stunted, prevented from assimilating food and inhibited in egg-laying by the precipitins. Present evidence is not conclusive on this subject, but several interesting suggestions have been made.

Before work on *Nippostrongylus* began, Blacklock, Gordon and Fine (1930) showed that death of the larvae of the myiasis-producing fly, *Cordylobia anthropophaga*, in the skin of immune guinea pigs is associated with a precipitate in the gut and around the larvae and that the precipitate in turn is due to the formation of precipitins by the immune host to the hemocele fluid and excreta of the larvae. They felt that this precipitate prevents assimilation of food and leads to death by

mechanically blocking the gut. Following the demonstration of a similar precipitate in *Nippostrongylus* by Sarles and Taliaferro (1936), Chandler (1937b) adopted a similar explanation for this nematode as a possible alternative to the one given below except that he believed the precipitinogens might be products of the digestion of the host's tissues. The *in vitro* studies of Sarles (1938) would indicate, however, that excretions and secretions of larvae which have never been in a host can act as precipitinogens. Taliaferro and Sarles (1939) believe the secretions of the esophageal gland are particularly important.

a. *Antienzyme antibodies.* The earlier suggestion given by Chandler (1935a, 1937b and 1939) postulated the acquisition of antienzymes by the host which inhibit the activity of specialized worm enzymes instrumental in digesting and assimilating host proteins. He homologized these antienzymes with the specific growth-inhibiting substances, suggested earlier by Schwartz, Alicata and Lucker (1931), and also with ablastin, the reproduction-inhibiting antibody, described by Taliaferro in infections with the *T. lewisi* group of trypanosomes (see review in Taliaferro, 1938). He (1937b) implied that these postulated antienzymes are similar to ablastin and different from true antibodies in lacking a marked physico-chemical affinity for the antigen and, hence, in being nonabsorbable with their specific antigens. As pointed out by Taliaferro (1938), his idea is almost identical with Ascoli's (1908) idea of antiblastic immunity in anthrax, especially as developed by Dochez and Avery (1916). There seems little doubt, on the one hand, that various immune serums do inhibit enzymatic and metabolic activity of bacteria and, on the other hand, that antienzyme antibodies can inhibit enzymatic activity. In both cases, however, the inhibition of enzymatic activity follows secondarily from the action of orthodox antiparasitic or antiprotein antibodies and not from immune principles with special characteristics. Thus, when antiserum is added to suspensions of bacteria and results in a decrease of metabolic and enzymatic activity, it would appear that the inhibition can be explained largely by agglutination which reduces the effective surface contact between bacteria and medium (see Blake, 1917). Similarly, when a specific antienzyme is added to purified enzymes in solution or to media in which the enzymes have diffused from the organisms and inhibits the enzyme activity, it would appear that the inhibition can be explained largely by precipitation which not only decreases the dispersion of the enzyme but removes it from the effective field of activity.

The work on enzymes may be briefly summarized as follows: The

results of Northrop (1930) and Seastone and Herriott (1937) with pepsin and pepsinogen, of Kirk and Sumner (1931) with urease, and of Campbell and Fourt (1939) with catalase demonstrate beyond doubt that highly purified, crystalline enzymes act as precipitinogens. Similarly, Ten Broeck (1934) has shown that crystalline trypsin, chymotrypsin and chymotrypsinogen will sensitize guinea pigs. In some cases, such as pepsin, as pointed out by Northrop (1930), the antigenic action may be small due to the rapid denaturization of the enzyme at the pH of the serum.

The clearest case of enzymatic inhibition is the demonstration by Kirk and Sumner (1931) that an antiurease antibody inhibits the hydrolysis of urea by urease both *in vivo* and *in vitro*. In reactions occurring *in vivo*, these authors found that rabbits can be immunized to withstand 100 times the ordinary fatal dose of urease and that this immunity can be passively transferred to rabbits and guinea pigs with immune serum. Kirk (1933) later showed that urease precipitated with this antibody and thoroughly washed with saline still retains about 80 per cent of its original activity, and he concluded that inhibition of enzymatic activity is due largely to decreasing the dispersion of the urease. Kirk and Sumner (1934) pointed out that the lack of inhibition cannot be due to a dissociation of the urease-antibody complex because the activity of the urease can be removed from urease-antibody mixtures by centrifugation and removal of the precipitate. They suggested, therefore, that the combination of antibody and antigen is such as not to affect that portion of the urease molecule responsible for urease activity. Much the same conclusions were reached by Campbell and Fourt (1939) who found that although an anticatalase antibody precipitates catalase in definite proportions, the catalase activity of such mixtures is almost unimpaired.

Briefly, therefore, the available evidence indicates that highly purified enzymes can act as precipitinogens. The resulting precipitins do not combine with the active radicle of the enzyme and do not specifically inhibit enzyme activity. They may, however, effectively inhibit enzyme activity secondarily by decreasing the dispersion of the enzyme and by removing it from its effective field of activity. It would seem likely that anti*Nippostrongylus* precipitins may act in this way. (See Smith and Lindsley, 1939, for a consideration of the same mechanism with antibacterial antibodies.) The fact that antienzyme antibodies are precipitins is contrary to the implication that they show no marked affinity for their antigen and, hence, are nonabsorbable with such antigens.

b. *Cellular phases of immunity.* The cellular responses, although secondary to antibodies in the immediate disposal of parasites in the immune animal, are of importance because 1, they lead to the worm nodules which surround immobilized parasites; 2, they give rise to increased numbers of macrophages which remove dead worms, and 3, they may detoxify foreign proteins (possibly the eosinophils) and may be instrumental in producing antibodies (probably the macrophages). In addition, Taliaferro and Sarles (1939) have studied the origin of macrophages in the nodules of the skin and lungs and, in particular, with respect to the functional significance of the lymphocyte in the production of macrophages. In the nodules formed in the skin, a few of the macrophages represent local members of the macrophage, i.e., reticulo-endothelial, system, but the great majority arise by the transformation of monocytes and lymphocytes which migrate from the blood stream. These findings are in agreement with the earlier work of Maximow (review in 1927) on local inflammation. In the lungs, more of the macrophages arise from the division of local macrophages (septal cells), but some arise as in the skin by the transformation of monocytes and lymphocytes which migrate from the blood stream. The functional significance of the agranulocytes, in particular of the lymphocyte, in giving rise to new macrophages is in line with earlier studies by Taliaferro and Mulligan (1937) on malaria and of Conway (1939) on certain bacterial infections.

4. *Ancylostoma.* One strain of *Ancylostoma caninum* occurs in the dog and another in the cat. After skin penetration it migrates as does *Nippostrongylus* except that its contact with the vascular system of the host in the lamina propria is more intimate (see review in Scott, 1930). Moreover, tissue migration is a hazard to developing larvae inasmuch as Sarles (1929b) found that only 9 per cent of infective larvae, if given cutaneously, develop in the intestine of puppies as compared to 50 per cent if given by mouth.

It is difficult to evaluate the rôle of age resistance of dogs and cats to hookworm as compared to acquired immunity in many of the investigations prior to 1931. McCoy (1931a) believed that an acquired immunity occurs in dogs and cats even though it may not be pronounced. Thus, dogs eliminate many of the worms present at the height of infection with *A. caninum* as well as prevent the development of additional larvae and throw off second and third infections more rapidly than an initial one. Although Foster (1935) found reason to doubt a true acquired resistance in dogs, his results can be interpreted otherwise, as pointed out by Otto and Kerr (1939). The latter investigators demon-

strated beyond doubt that subcutaneous doses of infective dog hookworm incite a relatively high degree of acquired immunity in dogs. Thus, 6 dogs withstood, with no pronounced ill effects, a subcutaneous superinfection of 120,000 or more larvae 31 to 60 days after the last of 26 subcutaneous injections with increasing numbers (15 to 30,000 or 60,000) of larvae, whereas 4 control dogs exhibited pronounced symptoms to an initial infection of the same number of larvae and 3 of them died 9 to 14 days thereafter.

A few years previously, Kerr (1936) reported an acquired immunity to *A. caninum* in the mouse. In this abnormal host, the worms migrate normally through the tissues, but are unable to establish themselves in the intestine. He found no retention or walling off of the larvae in the lungs or liver of normal or resistant animals, but found an acute inflammation in the skin and in the wall of the esophagus and stomach 24 hours after infection. This inflammation begins to disappear in normal mice by 48 hours, but in the resistant animals assumes the appearance of a chronic inflammation, is followed by a foreign-body reaction and eventually leads to the enclosing of the larvae in a fibrotic capsule. Although not demonstrated, he postulated an antibody which stimulates the local cellular response (cf. work by Kerr, 1938a, on *Ascaris* in the guinea pig and the role of antibodies in the allergic reaction to *Nippostrongylus*, as advanced by Taliaferro and Sarles, 1939). Later (1938b) he obtained suggestive evidence that the immunity can be transferred to normal mice with serum from mice previously infected with a series of sublethal doses of hookworm larvae and felt that an antibody may not only stimulate the cellular reactions but may also exert a direct effect on the larvae.

Chandler (1939) believes that the purely local intestinal phase of immunity, which he postulated for *Nippostrongylus*, is unimportant in hookworm immunity, and, hence, his views on hookworm immunity probably coincide with those of the reviewer on *Nippostrongylus* immunity. Available data are admittedly too meager to reach definite conclusions, but the studies of Kerr on the tissue reactions in the mouse as well as Otto's (1939) demonstration of a precipitin giving rise to precipitates around the mouth and anal opening of infective larvae *in vitro* indicate that the picture throughout infection with hookworm is probably similar to that as set forth by Taliaferro and Sarles (1939) in *Nippostrongylus*.

5. *Trichinella*. *Trichinella spiralis* undergoes its complete development in one host. The infective larvae are acquired by eating infected

muscle in which they are encysted. After digestion of the muscle in the stomach of the new host, the freed worms pass into the intestine where they may develop to maturity as early as 48 hours thereafter. After mating, the gravid females begin depositing larvae in about a week. The larvae reach the blood stream and become distributed by it over the body. They generally leave the blood stream in the muscles, move about freely for a time and then become encased in a thin membranous wall which is eventually calcified.

In 1921 Ducas reported that rats infected with *T. spiralis* develop an appreciable immunity to subsequent infection. This work, although not accepted by many at the time of publication, has since been confirmed by McCoy (1931b) and Bachman and Oliver González (1935). McCoy (1931b) greatly extended the results of Ducas by digesting entire animals and ascertaining the actual number of larvae in the muscles. He found that previously infected rats are able to withstand more than twice the dose of worms lethal to the majority of control animals. Only one-tenth as many of the swallowed larvae in the immune host develop to sexual maturity and even they are eliminated from the intestine before they deposit larvae. The immunity, therefore, is largely localized to the intestine. Further evidence of an intestinal "block" was obtained by Bachman and Rodriguez Molina (1933) in hogs and by Roth (1939) in guinea pigs. In studying the development of trichinae in abnormal environments, McCoy (1936) found that rats possessing an intestinal immunity to a second infection fail to inhibit the development of trichinae placed in the uterus. Later (1938) he ascertained that many of the worms eliminated from the intestine of immune rats are still viable—a fact which Chandler (1936a) had previously demonstrated in *Nippostrongylus* infections and which again emphasizes the temporary nature of many antiparasitic effects.

McCoy (1935) obtained various degrees of artificial immunization with repeated intraperitoneal injections of living trichina larvae, heat-killed larvae or dried, powdered larvae. In all cases the resistance in the artificially immunized rats did not affect the initial development of the adult worms in the intestine, but caused their more rapid elimination with a resultant smaller muscle invasion. Working with hogs, Bachman and Rodriguez Molina (1933) obtained no protection against trichiniasis after immunization by intramuscular injections of suspensions of trichina powder, and working with rats Bachman and Oliver González (1936) obtained similar results after feeding or after the intraperitoneal injection of dried powdered trichina. Spindler (1937) reported the

production of a certain amount of immunity in rats, rabbits and guinea pigs which were fed filtrates from trichinous rabbits after digestion.

In spite of earlier conflicting results, recent investigations indicate that serum from immune animals may be protective against infection. Salzer (1916) reported that convalescent serum from patients recovering from trichinosis exerts a protective and curative action. Schwartz (1917) questioned these conclusions and could find no evidence that serum from recovered animals exerts any effect on the larvae *in vitro* or influences the course of the disease when injected before or at the same time as feeding infective meat. It may be of significance, however, that Schwartz could find no evidence of immunity in his recovered animals and may, therefore, have been working with insufficiently immunized animals. Hall and Wigdor (1918) agreed with Schwartz that convalescent serum does not influence the development of the trichinae, but concluded that it does combat the toxic features of trichinosis since 9 out of 15 treated rats lived longer than the controls. Alexander (1923) reported that four 10 cc. injections of convalescent serum failed to influence the temperature, eosinophil count or duration of the disease in 2 human patients.

Trawinski (1935), on the other hand, reported that the subcutaneous injection of serum from heavily infected rabbits protects rats from 3 or more lethal doses of larvae, but concluded that such serum is antitoxic rather than antiparasitic because the treated animals survive, notwithstanding tremendous muscle infections. Nevertheless, there is at present no conclusive evidence that any of the helminths, including *Trichinella*, produces true antigenic toxins, and it is more probable that this so-called antitoxic effect is actually a retardation of the activities of the worm, possibly associated with stunting, immobilization and the prevention of food assimilation, as in *Nippostrongylus*. Culbertson and Kaplan (1938) found that serum from infected rabbits passively protects mice to the extent that approximately half as many infective forms develop to adults in the intestine of treated mice as in controls and the muscle invasion is less. According to these authors the antibody exerts its action on the ingested larvae maturing in the intestine rather than on the migrating or muscle forms since the number of larvae in the muscles is not lower than might be expected as a consequence of the decrease in number of adult worms in the intestine.

Chandler (1939) believes that in trichiniasis as in *Nippostrongylus* infection there is a general "parenteral" immunity and in addition a highly effective, purely local intestinal immunity. It seems to the

present reviewer, however, that the same general criticisms which have been previously given of his views on *Nippostrongylus* hold here. In fact, during at least part of the intestinal infection, *Trichinella* has a more intimate contact with the parenteral tissues than does *Nippostrongylus.* Heller (1933) found that the muscle stages, when fed to animals, excyst quickly and that their anterior ends can be found penetrating the epithelium and in the lamina propria of the gut, within 12 hours. Similarly, Bloom and the reviewer (unpublished data) have found worms deeply imbedded in the lamina propria of the duodenum of guinea pigs one hour after feeding worms freed from muscle. This intimate contact with the lamina propria (although the worms may later withdraw), together with the results of Culbertson and Kaplan (1938), would indicate 1, that Bachman and Rodriguez Molina (1933) and Bachman (1938) are probably correct in suggesting some type of antibody-antigen reaction during the intestinal phase of immunity; 2, that the local mobilization of leucocytes and cells of the reticulo-endothelial system, suggested by the same authors, is to a large extent the result of an allergic response as in *Nippostrongylus*; 3, that the allergic mechanism suggested by McCoy (1938) is secondary to the antigen-antibody reaction; and 4, that the inability of the worms to nourish themselves on immune tissue, as emphasized by Chandler, is probably also due to antibodies.

B. *Effective Antigens in Immunity to Infection.* The great diversity of antigens contained within a single worm is indicated by the comparatively sharp tissue specificity found by Canning (1929) in *Ascaris.* Only a beginning has, however, been made in ascertaining which antigens are involved in the stimulation of the antiparasitic factors in resistance to infection. The observations of Blacklock, Gordon and Fine (1930) and of Taliaferro and Sarles (1939) indicate that the effective antigens in immunity to infection are only a small number of the antigens actually present. The ineffectiveness of one antigen to stimulate actual resistance to infection, although stimulating the production of precipitins, is shown by the results of Campbell (1939a) on a polysaccharide which he isolated and purified from the larval form of *Taenia taeniaeformis.* He found that this polysaccharide is a complete antigen in that it stimulates the formation of precipitins *in vivo* as well as reacting *in vitro,* but is not of functional importance in immunity to infection because it does not immunize rats against infection, does not incite the formation of protective antibodies in rabbits and does not absorb the protective property from immune serum. Taken as a

whole, these results seem to indicate that the titer of a given antibody arising during infection need not parallel the degree of effective immunity and that caution is necessary in concluding from such a lack of parallelism that antibodies in general play no rôle in effective immunity.

CONCLUSIONS

The foregoing review of acquired immunity to the helminths is not intended to be complete, but is designed to indicate, in the few forms that have been most thoroughly studied, the facts that have been established and the more important ideas and differences in opinion of various investigators in an actively progressing field. As has been stressed by the reviewer (1934) and others, all evidence indicates that the various immunological mechanisms operative against the helminths, both humoral and cellular, are fundamentally identical with those operative against other infectious and noninfectious antigenic agents. Therefore the worms will undoubtedly be used with increasing frequency to solve those general problems in immunology for which they are best suited. Their chief advantages are connected with their large size, which makes it possible: 1, to study directly the effects of the immune mechanisms on the parasite and on parasitic metabolism; 2, to trace and locate the worms in the body, a characteristic which is particularly helpful in studying the immune mechanism *in vivo* and the cellular reactions of the host; and 3, to obtain large quantities of material, especially from the larger worms, for the preparation of antigens and for such chemical studies as the isolation of carbohydrates and enzymes.

In the specific field of helminth immunity, the following points are noteworthy: The evidence so far indicates that the mechanism of immunity rests immediately on humoral factors with secondary cellular coöperation. Precipitins, which are formed by the host against various materials passing out of the mouth, anus and excretory pore of the intestinal nematodes, result in visible precipitates *in vivo* and are unquestionably of functional importance in the allergic reactions of the host. It is probable, but not proven, that the precipitins are instrumental in affecting the immobilization, delay in development, stunting, prevention of food assimilation and inhibition of enzymatic activity of the parasite. In any case, humoral factors of some kind are operative. Two types of immunity, one "early" and the other "late," have been demonstrated in infections with larval tapeworms. Both can be correlated with antibodies, but whereas the antibodies of the "early"

type can be absorbed with freshly ground worm material, the others cannot. Further work is necessary on the nature of the antigens to the nonabsorbable antibodies, but evidence to date indicates that they are materials liberated from the living parasite and occur, if at all, in insufficient quantities in freshly ground worm material. The nature of the enzymes of parasites and the possibility of the functional inhibition of enzymatic activity by immunological antienzymes needs further study. The evidence so far indicates that the cellular responses, aside from their activity in producing antibodies, are secondary to antibody effects. The worm infections offer advantageous material for studying the local cellular reactions around parasites. They may also be used to study the effect of various factors, such as splenectomy and blockade of the macrophage system, which injure the connective tissue cells and thereby affect the cellular coöperation in immunity and the formation of antibodies.

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THE PITUITARY-ADRENOCORTICAL RELATIONSHIP

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Besides van Dyke's full reviews of this subject (309), several papers are available reviewing special aspects of it (92, 125, 130, 133, 285).

I. Effect of hypophysectomy. In the following animals, hypophysectomy is followed by a decrease in the weight of the adrenal glands: tadpoles (290), snakes (273), pigeons (253), mice (180), rats (43, 54, 58, 228, 237, 285, 291), rabbits (147, 318), and cats (218). Also in man, Simmonds' disease is accompanied by a decrease in adrenal size (57, 285, 289). The effect however is not seen so clearly in all species investigated; it is not seen in the toad (138) or the suckling pig (256). Although hypophysectomy often causes a decrease in the size of the dog's adrenals, the decrease is by no means as constant as in other species (38, 125, 136, 137). In the puppy, the adrenals cease to grow after hypophysectomy, but their histological structure remains essentially normal (59). In all of the species in which hypophysectomy is not followed by clear-cut adrenal atrophy, the operation does cause the decreased body growth and thyroid and gonad atrophy characteristic of the condition.

The adrenal atrophy is confined to the cortex, for the adrenal medulla retains for the most part its normal size and histology (58, 136, 137, 218, 245, 273, 291, 318). The cortical atrophy is rapid: in the rat the gland decreases to about one-half its normal size in six days (291); a similar effect is reported sometimes to occur in the dog (137). At about a month after operation, the atrophy is complete in the rat (54, 291), cat (218), and rabbit (318).

The histological changes concurrent with the gross atrophy are all in the direction of degeneration, particularly in the inner layers of the cortex. In the rat (54, 237, 245, 291), mouse (180), and dog (137), abrupt degeneration of the *zona reticularis* occurs first, with vacuolization and pyknosis. Similar degeneration in the *zona fasciculata* is soon apparent, so that thirty days postoperatively in the rat, rabbit, cat and dog this zone has largely disappeared (54, 137, 218, 291, 318). Meanwhile the Golgi apparatus has shrunken (245) and lipoid granules

are rare except in the middle portion of the cortex (180, 247, 291). The *zona glomerulosa* in both the rat (54) and the dog (137) is reported to undergo slight hyperplasia.

II. Adrenotropin. These gross and histologic changes in the adrenal cortex are prevented either by hypophyseal implants (43, 224, 273, 291) or by injections of suitable hypophyseal extracts (43, 76, 139, 225, 245). Such extracts will for convenience be designated *adrenotropin;* it is essentially the purpose of this paper to delineate the physiological actions of this substance or substances.

Adrenotropin has been found effective in the normal (72, 225, 309) as well as the hypophysectomized animal, causing hypertrophy of the cortex but not the medulla (61, 291). In normal rats, increases in weight of the gland of 300 per cent have been obtained (43, 61), and in hypophysectomized rats increases up to 700 per cent (61). It induces hypertrophy of all the cells of the cortex (61, 72, 139), except perhaps those of the juxta-medullary zone where the cells are senescent (245); it induces hyperplasia, particularly of the cells in the outer cortex (229); and it causes an increase in the size of the Golgi apparatus (245). It also produces a considerable formation of lipoid throughout the whole gland (72, 139, 229, 247), but more particularly in the outer *zona fasciculata* (229).

Various methods have been used in the preparation and concentration of adrenotropin (10, 43, 76, 198, 229). With a very potent preparation, one-eighth of a milligram has been found effective (43). It has been prepared free from many of the other pituitary trophic substances: from the growth-promoting hormone (229), from the thyrotropic hormone (43, 229), from the lactogenic hormone (253), and from the gonadotropic hormones (76, 229). Since many poisons or extracts may produce an hypertrophy in the normal, but not the hypophysectomized, animal's adrenal cortex (see below), the only specific assay object for adrenotropin is the hypophysectomized animal, the rat being usually employed (43, 76, 229). Others, however, have employed normal animals (rats, mice) for assay purposes (172, 229, 309).

Whether adrenotropin exerts its effects through the thyroid gland is a debated question, some (72, 186, 220, 258) reporting that a thyroidectomy prevents the adrenotropic action of pituitary extracts, while others deny it (16, 43, 139, 173, 229). Since adrenotropic extracts have been prepared free of thyrotropin, since hyperthyroidism in the hypophysectomized rat does not induce adrenocortical growth (297), and since adrenal growth can be shown to occur in the absence of the thyroid

(320, 328), it seems probable that the pituitary's control over adrenocortical size is not exerted through the thyroid. An "adrenotropic" action in pituitary extracts would be obtained by inducing hyperthyroidism by means of thyrotropin, and such an action would be prevented by thyroidectomy. This consideration may account for the controversial reports.

Adrenotropin is secreted only by the anterior lobe of the pituitary, since 1, removal of this lobe, without damage to the other portions of the gland, is followed by adrenocortical atrophy; since 2, removal of the posterior lobe alone does not cause appreciable changes in the adrenals; and since 3, implants of anterior, but not posterior, lobe exert adrenotropic effects (220, 291).

Histologists have attempted to ascribe to one type of anterior pituitary cell the production of a given trophic hormone, usually with controversial and inconclusive results. This appears true of adrenotropin as well, for some studies ascribe its secretion to the acidophils and some to the basophils. In pathologic material, adrenocortical tumors may accompany either basophilic adenomas (56, 117) or the acidophilic adenomas of acromegaly (57, 117), so these data are inconclusive. The clearest indication that one type of cell secretes adrenotropin is to be found in the hereditarily dwarfed mouse, in which, after spontaneous degeneration of the acidophils, adrenal aplasia occurs (292). Supporting this hint that the acidophils secrete adrenotropin are several other lines of evidence (276, 296). On the other hand, dwarf mice (21, 214) and thyroidectomized rats (320, 328), both having defective acidophilia, show normal adrenotropin reactions in some respects. This has been taken to indicate that the basophils must secrete adrenotropin; and various other studies support this conclusion (54, 285). Evidently the question remains open.

Several studies, none of them with very clear results, have been made by means of the implant technique on the pituitary's content of adrenotropin under various conditions (73, 221, 224).

III. Pituitary's influence on adrenocortical size. Very little is known about an hypophyseal control, if any, of the adrenal cortex during embryonic life. In the tadpole (290), but not the embryonic chick (321), the pituitary is essential for normal adrenal growth; in the mammal the available data, while very meagre, suggest that in fetal life the pituitary controls the growth only of the "fetal cortex" ("androgenic" zone) (9, 70, 103, 125; see 64) and that the rest of the gland does not fall under pituitary control until some time postnatally (59, 73

256, 292). Adrenotropin stimulates adrenocortical growth in the 4-day-old rat (338).

On the other hand, pituitary control of adrenocortical size in the adult and young adult has been clearly demonstrated. As shown above, hypophysectomy or pituitary extracts greatly influence adrenocortical morphology in the adult. Several other methods have been employed to demonstrate the pituitary's control of cortical growth; all of them show that stimuli normally producing growth are ineffective after hypophysectomy. These reactions include compensatory hypertrophy after surgical excision of most of the gland and growth after transplantation. Both are prevented by a previous hypophysectomy (43, 160, 285; 325). Another growth reaction in the cortex is the response to intoxication, trauma and other stresses: a vast number of stimuli produce this reaction (reviewed in 63, 103, 122, 278, 330)—in fact most disturbances in homeostasis cause adrenocortical hypertrophy and hyperplasia. (The pituitary is also profoundly affected by such stimuli (133, 278).) But after hypophysectomy the adrenal cortex appears entirely unable to respond; none among a variety of such intoxications had been found to cause any cortical growth (18, 71, 157, 277, 278, 299). That the stimulus to the pituitary in these reactions is a transient lowering of the available adrenocortical hormone is shown by the fact that adrenocortical extracts prevent all three of these types of cortical growth (160, 204; 160; 157). It has also been shown that such extracts, when administered in excess to the normal rat, cause adrenocortical atrophy (156, 161, 162), an effect prevented by pituitary extracts (161).

Much the same sort of experiments have been done with regard to the pituitary control of the gonads and the thyroid: hypophysectomy prevents compensatory hypertrophy of either gland (141; 16, 135, 207), and the "end-organ" hormone (i.e., the hormone produced by a gland influenced by the "central ganglion of the endocrine system"—the pituitary) of either gland causes an atrophy or resting state in its own gland (2, 222, 230; 36, 101, 119)—the familiar "suppressing" or "antagonistic" effect of the "end-organ" hormones on the pituitary. These experiments may be interpreted as follows: excess available "end-organ" hormone causes the pituitary to secrete less "end-organ"—stimulating hormone; hence atrophy of the "end-organ" gland occurs. And, vice versa, deficient available "end-organ" hormone causes the pituitary to secrete more "end-organ"—stimulating hormone; hence hypertrophy of the "end-organ" gland occurs. By such reciprocal means, a dynamic equilibrium is set up, resembling the dynamic equilib-

rium of reciprocal innervation of a muscle, in which the "end-organ" gland is supplied with a physiologic control of its rate of secretion, depending upon the needs of the organism. Such a concept when applied to endocrinological disease—e.g., Addison's disease—throws the onus of etiology on the pituitary; this hypothesis is, as yet, neither proved nor accepted.

IV. PITUITARY'S INFLUENCE ON ADRENOCORTICAL FUNCTION. Since *the pituitary is the "central ganglion" of the endocrine system, certain* of its actions are due to its influence on other glands. In order fully to prove such a relationship, it would seem that three steps must be taken: 1, excision of the pituitary must produce approximately the same effects (or, perhaps, less, see 129) as excision of the "end-organ" gland; 2, the "end-organ" gland's hormone must completely replace the hypophyseoprivic deficiency; and 3, suitable pituitary extracts must be shown inactive in preventing the deficiency after the "end-organ" gland is removed. If all three of these steps are successfully taken, then it is demonstrated that the pituitary controls the "end-organ" gland which in turn controls the process under consideration. For example, *estrus is prevented by either ovariectomy or hypophysectomy; estrogen* induces heat after hypophysectomy; but the gonadotropic substances are inactive after ovariectomy. Hence the pituitary controls the ovaries which in turn control estrus. If either of the last two conditions of proof is not fully met, then pituitary control is not demonstrated, with, *often, the possibility that the "end-organ" gland exerts its effect by* inducing a pituitary hyposecretion. For example, either hypophysectomy or adrenalectomy is followed by low fasting muscle glycogen levels (see below); but adrenocortical extracts will not entirely prevent this defect after hypophysectomy, and *pituitary extracts will prevent* it after adrenalectomy. Hence in this case, the fasting muscle glycogen *after adrenalectomy is low, probably* because the adrenals are not properly stimulating the pituitary.

This trifold proof of pituitary control over the adrenals is by no means completed. The available evidence indicates that in some cases its control is very slight, in others much greater, and in still others, although control is indicated, it is not yet fully proved. It is in this order that the pituitary's effects on adrenocortical function will be considered.

Solution of many of the questions *in this topic is, at the present writing*, impossible, largely because of several gaps in our knowledge of adrenocortical *physiology*. In the first place, preparations of the adrenocortical hormone (hormones?), which in this paper will be designated

by the abbreviation ACE, have only recently been widely available in potent form, and many negative reports of their use are probably due to poor extracts or to insufficient dosage or both. It is also probable that none of the present preparations furnishes a really complete replacement of the gland. For example, even with the best extracts, the observed symptoms of overdosage are very light (306, 307) in contrast to the effects of probable hypersecretion by adrenocortical tumors (219). (But see 336, 340.) In the second place, the fundamental question of how many hormones the adrenal cortex secretes cannot yet be answered. The gland appears to have a multiplicity of functions, but how they are interrelated, which lead to the others, and what fundamentally is the cause of death after adrenal ablation have not yet been solved. (See 335, 337.) In this paper the answer to these questions is not sought; rather, we are concerned with the one question: how much influence over adrenocortical functions does adrenotropin exert?

A. *Influence slight.* It is a widely held opinion that the primary cause of death in adrenocortical insufficiency is a disturbance in salt and water metabolism; reflecting this opinion is the common usage of the phrase "salt and water hormone" of the adrenal cortex, originally coined by Long and Lukens (190). This term is now customarily used to differentiate the functions of the cortex in salt and water metabolism from various other functions; although still an hypothetical differentiation, it is useful. The nature of the disturbance in "salt and water" metabolism that leads to death in adrenal insufficiency is still an open question which will not be discussed here; several critical reviews are available (107, 109, 110, 111, 113, 175, 185, 217, 301). It seems probable that the pituitary exerts only a slight influence over this adrenocortical function.

In the first place, the adrenals are necessary for life while the pituitary is not (except in the case of the fowl, which will be discussed below). Ablation of the former is followed by death in about a week, providing no therapeutic measures are taken, whereas ablation of the latter, although it does shorten the life span, is not immediately fatal. Furthermore, the changes which are most commonly associated with the defective "salt and water" metabolism of the adrenalectomized animal and which are thought to lead to death, are not observed in the hypophysectomized animal. These changes consist of disturbances in the blood Na, K, Cl, bicarbonate, non-protein nitrogen, and hydration. None of these constituents except K has been found changed after

hypophysectomy (129, 134, 211, 212). The blood K has been reported slightly lowered after hypophysectomy (211, 329), the reverse of the rise often (116, 331) but not always (304, 305) found in adrenal insufficiency.

Another effect of adrenal insufficiency, particularly in evidence in the rat, is a pronounced leaching out of many of the animal's minerals (259). This is accompanied by a considerable appetite for most of the common minerals (305). This salt-leaching effect is prevented by salt therapy (259) or life-sustaining diets (271). It is not, however, observed in the hypophysectomized rat (270), again an indication that hypophysectomy does not result in the same kind of defective "salt and water" metabolism as adrenalectomy.

It can be concluded, therefore, that adrenotropin does not exert the sort of absolute control over its "end-organ" gland that gonadotropin does. Evidently, the hypophysectomized animal's adrenals still secrete considerable "salt and water hormone." This is further proved by the fact that an adrenalectomy following hypophysectomy is followed by death with typical hypoadrenal symptoms (47, 269, 285).

As has been mentioned, fowls do not survive hypophysectomy (120, 227), although the pigeon does (216, 275). In fowls, while recovery from the trauma of the operation is rapid, about 80 per cent die within 48 hours and all die within about 10 days. The major symptom appears to be an abrupt asthenia, followed almost at once by death. It was also found (120) that this could be prevented by pituitary extracts or ACE, thus clearly indicating that the adrenals are at fault. In confirmation of this hypothesis is the report (118) that the same sort of symptoms and high mortality follow adrenalectomy in the fowl.

Although in all species except the fowl, the adrenals of the hypophysectomized animal appear to secrete enough "salt and water hormone" to maintain life, nevertheless, there is some evidence showing that they do not secrete it as efficiently as the normal animal does. Neither the adrenalectomized nor the hypophysectomized (94, 286) animal excretes water properly after it is injected intraperitoneally, a defect prevented in the hypophysectomized animal by ACE (94). Also, neither the hypophysectomized animal, treated with adequate lactogenic hormone, nor the adrenalectomized animal, is able properly to lactate. This deficiency is corrected in both types of animal by ACE (92, 93, 213).

Besides these two conditions in the hypophysectomized animal in which the "salt and water hormone" of the adrenals appears to be

insufficiently secreted, there is still a third. The reaction of the organism to the shift in body water and electrolytes that is produced by the administration of isotonic glucose solution intraperitoneally (60) has been much used in studying adrenal insufficiency (97, 108, 287, 303). The injection produces even in normal animals the blood changes of adrenal insufficiency, including those in Na, K, Cl, and protein; hence, it is urged, the fundamental disturbance in the two conditions must be similar. The performance of this experiment on the hypophysectomized animal might be expected to furnish a good test as to whether the salt and water hormone were properly secreted after hypophysectomy. This has been done (94), but the results are so complicated by other factors that they are not conclusive. The reader is referred for details to the interesting original paper; in *some* aspects, the reactions of the hypophysectomized rat resembled those of the adrenalectomized rat and in *some* cases ACE prevented the defects observed in the hypophysectomized rats. In summary, the adrenals of the hypophysectomized rat clearly secrete sufficient "salt and water" hormone to maintain life, but in some stresses, it has been shown that this hormone is not secreted entirely adequately.

A relation of adrenotropin to diabetes insipidus has been postulated (288). Since removal or denervation of the posterior hypophysis leads to a permanent diabetes insipidus, but total hypophysectomy leads only to a transient diabetes insipidus, the anterior lobe is thought to secrete a "diuretic" hormone (80, 248). Although some experiments indicate this hormone to be associated with thyrotropin (80, 208), recent evidence minimizes the thyroid's participation in this reaction (80, 299). It is possible (288) that the "diuretic hormone" is associated with adrenotropin, and it has been reported (49) that the transient diabetes insipidus following total hypophysectomy in the rat is prevented by a concurrent adrenalectomy. However, the permanent diabetes insipidus of the cat with hypothalamic lesions is not prevented by adrenalectomy, particularly if the animal is given saline therapy (164, 165). Although it is evident that the "diuretic hormone" of the anterior lobe has not yet been conclusively shown to be adrenotropin, it is to be anticipated that the adrenal cortex and the *pars nervosa* will be shown to be intimately related, because ablation of either leads to defective NaCl metabolism (see above and 297a).

A slowed absorption of glucose has been observed in the adrenalectomized rat (7, 41, 50, 319) and cat (166), but not the toad (144); and a similar defect occurs in the hypophysectomized rat (22, 243, 262, 268).

Since, however, ACE does not restore the absorptive ability of the hypophysectomized rat entirely to normal (82), while thyroxin does (262), it is evident that adrenotropin is not the only agent involved. Significantly also, the defective absorption following adrenalectomy is prevented by saline administration (7, 41, 67). Considering all these facts together, the absorptive defect of adrenalectomy appears due to the lack of "salt and water" hormone, while that of hypophysectomy appears due to several factors, among which is certainly thyrotropin. But whether adrenotropin is also involved is not yet clearly established. The other defects in absorption that follow adrenalectomy cannot as yet be related to the pituitary, for the effect of hypophysectomy on them has not yet been determined.

A considerable anorexia occurs after hypophysectomy as well as adrenalectomy. In the case of the rat, either preparation shows a greatly reduced food intake (182, 210, 259), with a negative caloric balance (182). But this reaction also appears to be associated in the one case with the "salt and water" hormone and in the other with non-adrenotropic factors, for adequate salt therapy induces a positive caloric balance after adrenalectomy (3, 259) and ACE does not prevent the weight loss following hypophysectomy in the rat (15, 154, 285). Grollman and his associates (105, 285) have suggested that the growth defects following adrenalectomy are in large measure due to a resultant hyposecretion by the pituitary. It seems more probable that inanition and poor absorption are the direct cause of the condition, because good growth has been obtained after adrenalectomy in either the rat (259) or the dog (3) with saline therapy alone.

Finally, either type of operation in the rat decreases its glucose tolerance (264, 267). But since salt therapy restores normalcy after adrenalectomy and since ACE is ineffective after hypophysectomy (269), this reaction also appears subject to the conclusions drawn above.

B. *Influence partial.* Those reactions in which adrenotropin appears to exert only minimal control over the adrenals have been discussed; they are: the adrenals' "vital function" and their effects on dextrose absorption, appetite, and sugar tolerance. There are, however, several reactions in which adrenotropin appears to exert partial, if not complete, control of adrenocortical functions. One of these is the adrenals' participation in the processes tending to protect the animal against intoxication, trauma and many such stresses.

The sensitivity of the adrenalectomized as well as the hypophysectomized animal to even mild stresses is generally recognized: to heat (112, 317; 291), or cold (17, 123, 255, 295, 317, 323; 17, 278, 291) or

muscular exercise (277, 278), to mention a few ordinary ones. Similarly both are very sensitive to trauma (84, 277, 278, 300; 133, 277, 278), hemorrhage (302; 132, 133), infection (277, 278; 133) or toxins of many sorts (52, 103, 184, 277, 278, 308; 133). Special conditions, it must be added, may modify these reactions (see 278). In these studies, frogs, toads, rats, cats and dogs have been commonly employed. Further demonstrating the participation of the adrenals in these reactions, are its histologic response to intoxication, which has been discussed above, and the reports that "cortin" excretion in the urine is increased in illness (315) and in the trauma of surgical operations (316).

With respect to intoxication, certain species differences, however, and certain differences in the results produced by the two operations are apparent. The adrenalectomized toad (98) but not the adrenalectomized frog (1), appears to undergo but little change in sensitivity. On the other hand, the hypophysectomized toad is quite sensitive to intoxication (128, but see 98). The adrenalectomized dog or rat is very sensitive to trauma, infection, and illness, but by contrast the hypophysectomized dog or rat is quite resistant, tolerating readily, for example, in the case of the dog, a pancreatectomy. In general, hypophysectomy in the mammal does seem to decrease resistance less than adrenalectomy.

In the rat, quantitative data for the effects of the two operations on resistance to histamine-poisoning are available. After either operation, the lethal dose is reported to be reduced to about the same order of magnitude (52, 152, 238, 322). The adrenals' functions in this reaction are not cortical only, for the presence of the medulla is necessary for some of the resistance detectable in the normal animal and adrenalin alone confers some resistance in the adrenalectomized rat (152, 240, 322, 324).

Whether saline therapy will mitigate materially these types of intoxication has not yet been quantitatively measured. The adrenalectomized dog, maintained on saline therapy, is still an exceedingly fragile animal (3, 5, 300). On the other hand, the rat in these conditions is in fairly good health, being able to stand various intoxications (84, 277, 278, 317) far better than the untreated adrenalectomized rat.

In the critical experiment of determining the effect of ACE on the hypophysectomized animal's resistance to intoxication, it has been found that ACE restores the resistance of the animal (rats), partially in the case of histamine (238, 239, 241) and entirely in the cases of formaldehyde or adrenalin poisoning (277).

In summary, the resistance of the animal to intoxication and stress

appears to be under partial control of the adrenotropin-adrenocortical system. There appear, however, to be considerable species differences in the degree of adrenotropin control. Further comparative and quantitative studies are desirable.

Another line of evidence showing the participation of this same system in the organism's reactions to intoxication is to be found in the data showing its control over the lymphatic tissue throughout the body. The morphology of this tissue, including particularly the thymus in both its cortical and medullary portions, is profoundly changed in stresses like intoxication, malnutrition, fasting, etc., etc. (106, 277, 278), involutionary, and other changes being very apparent. These reactions are almost entirely prevented by adrenalectomy (181, 277, 278) and partially prevented by hypophysectomy (277). How these changes are related to resistance to stress is still very obscure; that they cannot be primarily and solely responsible for resistance is indicated by the report that although involution is prevented in the adrenalectomized rat, treated with saline and then submitted to stress, such rats are nevertheless quite resistant to intoxication (277).

In the absence of the adrenals, not only is lymphatic involution prevented, but also thymic and lymphatic tissue hyperplasia occurs (27, 55, 103, 168, 177, 278). The effects of hypophysectomy on this reaction are controversial (140, 148, 250, 278, 309). Thymic shrinkage has been produced in the normal, adrenalectomized or hypophysectomized rat with ACE, given in large doses (153, 162, 277, 278).

From these experiments, it is clear that the morphology of the lymphatic tissue is vastly influenced by the adrenal cortex. Adrenotropin exerts probably somewhat less, but nevertheless a very great, control over this tissue. It must not, however, be considered controlled only by the adrenotropin-adrenocortical system, for both hyperthyroidism (35) and gonadectomy (86, 278) lead to its hypertrophy. The gonadotropic (33, 77) and many of the gonad (40, 148, 176, 274) hormones also induce atrophy. Since the thymus of the gonadectomized rat, but not that of the adrenalectomized rat, atrophies in intoxications (278), it seems probable that the adrenals are more closely related to the phenomenon than the gonads.

C. *Influence great.* The pituitary and adrenals are also involved in intermediary fat, protein, and sugar metabolism. Participation in fat metabolism has been demonstrated in various ways. After hypophysectomy as well as after adrenalectomy, fatty infiltration of the liver as caused by phosphorus poisoning (312; 167), or partial extirpation of the

liver (279; 203, 279) or pancreatic diabetes (191; 191) is prevented. Chaikoff, Gibbs, Holtom and Reichert (37) dissent from the latter conclusion, but since both of their surviving two dogs showed only slight ameliorations of the diabetic state after hypophysectomy, their operations were presumably incomplete. Since ACE induces in the phosphorus-poisoned, hypophysectomized rat a considerable fatty infiltration (167) and since the fatty infiltration produced by certain pituitary extracts is prevented by a previous adrenalectomy (87, 202), it is clear that the phenomenon of fatty infiltration of the liver is controlled by the adrenotropin-adrenocortical system.

Since Rietti's first report (254) that hypophysectomy prevents the ketonuria caused by pancreatic diabetes, there has been a great number of confirmations, in various species (130, 194, 260). It has also been shown that adrenalectomy largely if not entirely arrests the ketonuria of the pregnant rat (199) and of the diabetic dog or cat (187, 196, 260). Similarly, the ketonuria of phlorizin poisoning is largely prevented by either operation (24, 74). The same type of study has been made, although incompletely, of the ketone bodies in the blood. Hypophysectomy prevents their appearance in pancreatic diabetes (130) and adrenalectomy is reported to prevent the ketonemic action of pituitary extracts (143, 201). The interpretation commonly given to all of these facts is that the control of fat metabolism by the pituitary is carried on through the adrenals. If this is true, then adrenocortical extracts should be ketogenic in the normal animal. MacKay (200) has claimed this, but Grollman (104) denies it.

Although it is generally agreed that adrenalectomy prevents most experimental *ketonurias*,[1] several recent reports (206, 226, 233) have denied that the *ketonemia* produced by pituitary extracts is prevented by adrenalectomy. It has also been reported that adrenalectomy does not prevent the ketonemia of fasting (206, 233). It is evident that the ketonuria of a given preparation cannot be taken as a measurement of ketogenesis, for any observed changes may be due to changes in the kidney thresholds for ketone bodies. If these studies are confirmed, a part, but obviously not all, of the widely accepted conclusion that the "ketogenic" actions of the pituitary are carried on through the adrenals, will have to be revised.

The relation of the pituitary to protein metabolism has been recently

[1] This has been denied for the ketonuria produced by pituitary extracts (333, 341).

reviewed by Houssay (129). In the dog the great increases in protein catabolism induced by feeding protein-free diets, by pancreatic diabetes, and by phlorizin poisoning are all much dampened by hypophysectomy, because in these conditions it is found that total nitrogen excretion is reduced by about one-half. Further studies on pancreatic diabetes (187, 189, 196, 260), phlorizin poisoning (74), and the protein catabolism induced in the rat by low atmospheric pressures (75) have in general confirmed Houssay's conclusion that "the pituitary gland is an important regulator of endogenous protein metabolism, which it stimulates. . . . It also takes part in the formation of sugar from protein." Houssay also states: "The lowered protein catabolism (following hypophysectomy) cannot be explained by hypothyroidism because, although thyroidectomized dogs have a decreased nitrogen excretion in simple or protein fasting, when there is need of large protein destruction (e.g., in pancreatic or phlorizin diabetes) this is as exaggerated as in the controls, whereas the increase is very small in hypophysectomized animals." The thyroid and thyrotropin do not therefore appear to be directly involved in these reactions (but see 294); rather, since Houssay's review, considerable evidence has accumulated to show that the adrenals and indirectly adrenotropin are the agents involved. The rapid nitrogen excretion of pancreatic diabetes in the dog, cat, and rat (39, 187, 260), that of phlorizin poisoning (74), and that of hyperthyroidism in the dog (177) are all much reduced, if not prevented, by adrenalectomy, just as they are by hypophysectomy. The necessary final step in the proof—that of measuring the effect of ACE in the hypophysectomized animal submitted to these stresses—has been taken, with negative results, in the case of phlorizin diabetes (75). But in the case of pancreatic diabetes, it has been shown (196) that the depancreatized-hypophysectomized cat treated with ACE, excretes more nitrogen than the untreated animal. Presumably with proper dosage and better preparations of ACE, the same will be found true of the other stresses. In summary, adrenotropin exerts a profound effect on endogenous protein catabolism through its control of the adrenal cortex.

Although it is evident that adrenalectomy or hypophysectomy do slow up either fat or protein catabolism, it is also clear that neither one of the glands is essential to these processes; rather, both processes can and do go on in the absence of adrenals or pituitary. This is shown, first, by the fact that both preparations lose weight, often becoming very thin before death. Furthermore, in investigations of the composition of the weight loss following hypophysectomy in the rat (182),

though fat catabolism is slowed, it is present; protein catabolism also occurs to a considerable extent (14, 182, 242). Although, therefore, both processes continue without these glands, it seems that they go on very sluggishly—so sluggishly in fact that they are not able to sustain the animal's fuel needs in simple stresses like fasting (293) and much less so in great stresses like insulin shock. Of the two great reserves of fuel, fat and protein, the control by the adrenotropin-adrenocortical system over fat catabolism appears more complete than that over protein catabolism (182). Sustaining this conclusion is one interesting report that even in hyperthyroidism, the hypophysectomized rabbit is depleted of fat slightly if at all (45).

Given an animal in which neither fat nor protein is readily available for energy, one might infer profound derangements of the carbohydrate metabolism. Precisely this happens after hypophysectomy as well as adrenalectomy. This subject, particularly in reference to the pituitary, has recently been reviewed in several comprehensive reports (121, 130, 183, 187, 194, 260, 309). (See also a recent symposium (343).) In this paper, only a short summary of recent work will be attempted. References quoted are primarily to review or to recent articles.

In its carbohydrate metabolism, many reactions of the organism are common to either hypophysectomy or adrenalectomy. In both, there is a tendency to fasting hypoglycemia, marked after hypophysectomy, in both the fasting liver glycogen is much reduced, in both the fasting muscle glycogen is very low, and both are very sensitive to insulin (130, 187, 194, 260). In both the glycogen stores increase but little in response to low atmospheric pressures (75), and in both the severity of phlorizin diabetes is markedly reduced, there being little or no glycosuria (74, 130). The ameliorating effect of hypophysectomy on pancreatic diabetes is firmly established; the effect of adrenalectomy on pancreatic diabetes is also clear: it ameliorates the experimental condition, to much the same extent as does hypophysectomy, in the toad (142), rat (104, 187, 193), cat (39, 78, 115, 187, 196), and dog (145, 187, 196). Neither salt therapy nor the presence or absence of the adrenal medulla appreciably alters these reactions (187, 298, but see 51).

But proof as to how many of these reactions are due to the adrenotropin-adrenocortical system has been difficult and only recently successful. Since ACE was often not found, in the earlier work, to produce a reversal of the effects of adrenalectomy or hypophysectomy (142, 187, 194, 260) and since it has never been found to be as diabetogenic as pituitary extracts (104, 142, 145, 196), the hypothesis that all the

pituitary's effects on sugar metabolism were due to adrenotropin was never clearly established. Very recently, however, ACE, given in very frequent doses, has been found to induce diabetes in the adrenalectomized-depancreatized dog or cat (196), in the adrenalectomized-partially depancreatized rat (88, 104, 193) and in the "Houssay" cat (196), thus in some measure confirming the theory. And yet, the case is not clear since pituitary extracts are still diabetogenic, although perhaps reducedly so, in the adrenalectomized-depancreatized animal (145, 187, 194). This has led to the hypothesis "that the anterior pituitary can influence carbohydrate metabolism a, by the action of the adrenotropic hormone in stimulating the activity of the adrenal cortex, and, b through another factor (or factors) that exerts its effect directly on the tissues" (88).

Confirmation of this hypothesis is furnished by other studies of the effects of ACE on sugar metabolism. Taking the clearest possible case, that of fasted hypophysectomized rats, it has been shown (Grollman dissenting, 104) in these that frequent ACE administration induces a great increase in liver glycogen (48, 83, 174, 195, 265) and a return of the blood sugar to normal or mildly hyperglycemic levels (48, 174, 195, 265). But this treatment does not restore the depleted muscle glycogen to normal (195, 174, 265), and it cannot be said to be very hyperglycemic (48, 104, 253, 265).[2] The deposit of muscle glycogen, instead, appears controlled by an extra-adrenotropic factor, for it can be greatly increased by injecting pituitary extracts into the adrenalectomized rat (23, 263). Besides these hormonal studies, the effects of the two operations on muscle glycogen differ in their timing: whereas hypophysectomy leads at once to defective muscle glycostasis, adrenalectomy leads to it only several days postoperatively (23). Such experiments strongly indicate that following adrenalectomy muscle glycogen levels are poorly maintained in fasting and stress (30, 194) because of a pituitary hyposecretion induced by the adrenalectomy. Exactly how the excessive sugar utilization of the fasted hypophysectomized rat (81, 223, 261) is related to adrenotropin and the adrenals is not yet clear; in one recent report, Russell (263) considers that both are involved. The relation to the adrenal cortex of the other multitudinous—and protean—types of action of pituitary extracts on carbohydrate metabolism is still an open question (66, 187, 233, 295, 326, 327). That all the pituitary's controls over carbohydrate, fat, and protein metabolism follow directly

[2] Confirmation (334, 337, 339, 342), along with the suggestion of synergistic effects by the two glands (339), has recently been obtained.

from two or three fundamental processes is a very attractive, but still controversial, hypothesis (187, 284, 326).

In summary, evidence that many of the changes in carbohydrate metabolism following hypophysectomy are due to the resultant adrenocortical hypofunction is rapidly accumulating. The old clinical observation (219, 283) of diabetes in association with some adrenocortical tumors thus receives experimental support. It must be emphasized that although at this present writing some aspects of the pituitary control appear adrenal and others extra-adrenal, the distinction is still none too clear, being, in some but not other regards, a matter of degree rather than of kind. For example, ACE restores, in the hypophysectomized rat, a modicum of muscle glycogen, although it does not restore it to normal or super-normal. The conclusions drawn are only tentative; they require amplification, and, particularly, application to other species than the rat. But the present status of the subject furnishes the answer to several troublesome questions that have hitherto been unexplained; it appears that the carbohydrate metabolism after adrenalectomy is less defective than that after hypophysectomy because the pituitary after adrenalectomy is still functioning fairly well. After adrenalectomy, pituitary extracts are found active in carbohydrate metabolism by some workers but not by others because some extracts contain primarily the adrenotropin-mediated activities and others nonadrenal factors. But there are still many points to be settled. For example, from what precursors in the adrenalectomized rat treated with pituitary extracts does the muscle glycogen come, since after adrenalectomy neither fat nor protein is readily broken down?

Many investigators have in mind the possibility that the thyroid participates in these reactions. Its influence, however, appears light in comparison with that of the adrenal cortex (19, 69, 130, 187, 194, but see 294), particularly from Russell's (262) study.

D. *Influence suspected.* The control by hypophyseal adrenotropin over the "vital" function of the cortex, over absorption, and over anorexia has been shown to be minimal, in contrast with the intimate control exerted over intermediary fat, protein and sugar metabolism. There are also certain adrenal reactions which the pituitary probably influences greatly, but which have not yet been adequately established.

Another consequence of adrenalectomy as well as hypophysectomy is asthenia and susceptibility to fatigue, demonstrable with a variety of technics (27, 90, 114, 149; 59, 154, 155, 272, 314). The onset of this condition is rapid, occurring in the rat within twenty-four hours after

either operation (*149, 154*); the adrenal medulla is not apparently involved (159). The asthenia of hypophysectomy appears to be greater than that of adrenalectomy, for the former operation reduces performance to about 3 per cent of normal whereas the latter reduces it to about 15 per cent of normal (151, 154, 155). Other types of activity in the organism, both entailing work by the skeletal muscles, are similarly reduced by either operation, i.e., *spontaneous activity*, as measured by the revolving drum technic, and the ability to withstand exposure to cold (15, 150, 249, 250; 17, 123, 255, 278, 281, 291, 317, 323). In all of these, salt therapy has but slight ameliorating effects (249, 317).

The effect of ACE on the hypophyseoprivic animal's behavior in these situations is, with one exception, minimal. It prevents none of the asthenia of the hypophysectomized toad (126) and none of the susceptibility of the hypophysectomized rat to fatigue in swimming (314). It restores some of the hypophyseoprivic rat's "liveliness," but increases only very slightly its spontaneous activity (15). It does produce an appreciable but slight resistance to fatigue in ergographic tests on the rat, raising performance from an operative low of about 3 per cent of normal to 22 per cent of normal (154). All of these data point to minimal control by adrenotropin over the hypophysectomized animal's susceptibility to muscular fatigue. However, one report entirely dissents from this conclusion. The great susceptibility to cold of the hypophysectomized rat is said to be entirely prevented by ACE (17). On the other hand, the hypothermia of hypophysectomy is reported not prevented by ACE (105).

Considerably more experimental work on this topic is desirable, since obviously some types of hypophyseoprivic asthenia appear due to the resultant adrenotropin deficiency, others less so, and still others not at all. Studies of the lowered muscular efficiency that follows either operation have not yet clearly shown its cause. The effect of the two glands on muscle glycogen has been discussed and it seems likely that this is the main cause of the asthenia. The great reduction of available muscle fuel in either preparation would certainly be expected to lead to a great asthenia. Furthermore, ACE restores the muscle glycogen after adrenalectomy (30, 31), and it abolishes the asthenia as well (114, 151). In the hypophysectomized rat, ACE restores neither the muscle glycogen nor, in large part, the asthenia (see above). On this basis, to restore full muscular efficiency in the hypophysectomized animal, a combination of adrenotropin (or ACE) and the pituitary "glycostatic" factor would be necessary. This has been shown true to some extent.

Crude pituitary extracts are reported to prevent the asthenia of the toad (128), and to restore, sometimes to normal, the rat's spontaneous activity (249). However, they were inactive in restoring the rat's ability to withstand the fatigue of swimming (314). Also, adrenotropin is no more effective than ACE on ergographic performance, and relatively pure preparations of mammatropin, thyrotropin, or the growth promoting hormone exert only minimal effects (163).

Besides the changes in muscle glycogen after either operation, various other muscle compounds have been studied. Muscle phosphocreatine is reported reduced by either operation by some (32, 44, 178, 209, 231, 234) but not by others (89, 197). Lactic acid production is also reported reduced (44, 209, 232, 313) or unchanged (89, 124). Prevention with ACE of some of these defects in the hypophysectomized animal has been attempted, with negative results (127).

The relation of the pituitary gland to blood pressure was fully reviewed by Houssay (132) in 1936; in this paper his review will only be brought up to date. The possibility of some connection between the basophil cells of the pituitary and hypertension has aroused continuous interest (56, 117) but has not been clearly demonstrated in pathologic material (244). Experimentally the relation has received much support. Adrenalectomy largely prevents the hypertension produced by renal ischemia (25, 42, 68, 99, 235; one dissenting report, 257) and that produced by intracisternal kaolin injections (169). Neither the medulla (6, 100, 235) nor salt therapy (68, 99, 235) participate in these effects to any great extent. Hypophysectomy is also reported (236) partially to arrest experimental hypertension. The critical experiment of treating the hypertensive hypophysectomized animal with ACE has not yet been performed; moreover, neither ACE nor pituitary extracts have yet been shown to be truly hypertensive in either the normal or the adrenalectomized animal with ischemic kidneys (42, 99, 235).[3] An extract of hypertensive blood is said to be adrenotropic (170) and of hypertensive urine to contain "cortin" (315). One interesting report has been made (26) that the establishment of excessive pituitary secretion in the amphibian, *Amblystoma*, by pituitary transplants is followed by various signs of hypertension in kidneys, heart, and skin. These data are all strongly suggestive, but do not as yet furnish full proof of the hypothesis that the adrenotropin-adrenocortical system are involved in hypertension.

[3] Two recent reports suggest that this will soon be adequately demonstrated (336, 344).

The common embryonic origin of gonads and adrenal cortex, the history of the curious "X-zone" or "androgenic zone" in the ontogeny of some species (64, 103) and in pathology (103, 117), the many overlapping effects of extracts of the two glands (34, 46, 95, 96, 102, 109, 158, 246, 266, 274, 310, 311), and other experimental relations of the two (61, 62, 118, 146) all point to some sort of gonadotropic or gonad hormone action by the adrenals and *vice versa*. That the pituitary controls these reactions seems very probable (61, 62, 64), but what their meaning is in normal physiology is still obscure. In the fowl, the adrenals appear essential to reproduction (118), but in the rat (91) and the dog (4), all the reproductive processes up to lactation may be normal in the absence of the adrenals, providing adequate saline therapy is given. The defective lactation appears to be due, as has been shown above, to deficient secretion of the "salt and water hormone."

V. Adrenocortical influence on pituitary function. The pituitary, as is now generally recognized, is the "source and target of active substances" (85). No doubt, the adrenocortical hormones profoundly affect pituitary secretion rates, but these effects remain largely undefined. The following papers discuss various points (46, 53, 65, 125, 171, 215, 285).

VI. Discussion. Adrenotropin may be said to have the following functions: it controls the adrenal cortex's activities in sugar, fat, and protein metabolism, and, to some extent, its activities in protection of the organism against intoxication. It is probable that it controls the adrenals' functions in muscle metabolism, in experimental hypertension, and in some species, reproduction. The adrenals' vital functions and their influence on sugar absorption and appetite—those which are corrected to a large extent by salt therapy—appear only minimally controlled by the pituitary. Presumably, the portions of the cortex only slightly affected by hypophysectomy, i.e., primarily the glomerular layer, secrete the "salt and water" hormone. This is in keeping with the following generality: those processes which are necessary for life are, to a degree, autonomous; although they may be profoundly influenced by the correlating systems of the organism, they can function independently. Thus, the parathyroids, the internal secretions of the pancreas, and the outer layers of the adrenal cortex are largely independent of control by the "central ganglion of the endocrine system," the pituitary.

Since hypophysectomy leads to degeneration of the internal layers of the cortex, it seems plausible to ascribe to them the adrenal secretions not produced after hypophysectomy, i.e., the sugar metabolism and

other effects. This suggests that the adrenal cortex secretes two hormones at least: one, the "salt and water hormone" and the other(s), those that influence sugar metabolism, etc. But there are other possibilities. One is that the hypophysectomized animal's adrenals secrete only enough hormone to maintain its "salt and water" functions, but not enough beyond this quantity to maintain its sugar metabolism and other functions (see 79). Another possibility is that the autacoid produced by the outer layer, as it passes in the normal animal down the adrenocortical sinusoids toward the medulla, is worked over—chemically changed—by the fasciculate and reticular layers so that it acquires, in addition to its "salt and water" functions, the other functions of the cortex. Now in the normal animal, the two inner layers of cells are derived from the outer layer (160a)—in fact, the adrenocortical cells are thought slowly to move from the capsule toward the medulla, eventually dying at the juxtamedullary zone (see 122). In the hypophysectomized animal, however, the internal layers of the cortex have degenerated; they are not present to work over—or "ripen"—the hypothetical secretion of the outer layer. The hypophyseoprivic animal secretes only an "immature" hormone. The function of the pituitary, then, would be to preserve the lives and secretory activities of the adrenocortical cells in the fasciculate and reticular phases of their history.

SUMMARY

The control exerted by the pituitary over the adrenal cortex appears great in some aspects, partial in some, and minimal in others. The data are best summarized in tabular form:

ADRENOCORTICAL FUNCTION	DEGREE OF PITUITARY CONTROL
Vital function, i.e., relation to "salt and water" metabolism	Slight but demonstrable
Dextrose absorption	Slight but demonstrable
Anorexia and inanition	Slight
Resistance to the stresses of intoxication, trauma, etc., etc.	Partial
Sugar metabolism	Probably complete
Fat metabolism	Probably complete
Protein metabolism	Probably complete
Muscle metabolism	Suspected but not yet clearly proved
Experimental hypertension	Suspected but not yet clearly proved
Reproduction in some species	Suspected but not yet clearly proved

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RECENT ADVANCES IN THE CHEMISTRY OF CALCIFICATION

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Whether or not the inorganic matter of bone will be deposited in adequate amounts together with an organic matrix of the usual shape and texture is known to be influenced by the following factors: 1, the endocrine glands, especially the parathyroid, the thyroids, the pituitary and the sex glands; 2, the vitamins, particularly vitamins C and D; 3, the supply of calcium and phosphate as compared with the daily requirement, and 4, in certain species, at least, the acid-base balance of the individual.

It is the purpose of the present review to correlate the recent information which is pertinent to these four categories.[1]

An inadequate supply of calcium and phosphate to an otherwise normal animal obviously has its effect on bone itself. The places of action of the other three groups of factors are often not so obvious. These factors may change the course of calcification by their influence directly or indirectly in one or more of the following ways: 1, by acting directly on the bone cells and thereby altering the rate of deposition and solution of the inorganic and organic solids; 2, by varying the net absorption of calcium and phosphate from the intestinal tract, or 3, by regulating the urinary excretion of these constituents.

Much of the work of the last few years has been directed toward finding the locus of action of the factors known to be physiologically active in calcification, and toward defining their action in the different tissues. To this end, efforts have been made to determine whether the bone salt can form spontaneously from blood plasma. The answer to this question would determine 1, whether it is necessary to assume that the bone-forming cells are required to increase the concentrations of

[1] Since, in 1935, there appeared a comprehensive review of calcium by Schmidt and Greenberg, this review will be confined largely to contributions made since that time.

calcium and phosphate locally in order to initiate precipitation, or 2, whether sufficiently high concentrations of calcium and phosphate in the body fluids have already been provided by the balance between absorption and excretion. Three lines of recent research bear on this point—namely, 1, application of the Mass Law to the ionization of calcium salts and of phosphates in the blood plasma; 2, investigation of the nature of the bone solid by chemical analysis and x-ray spectrography, and 3, a study of the steps involved in the formation of the bone salt.

Calcium ions in body fluids. Rona and Takahashi (1913) were the first to give experimental evidence that part of the calcium of the blood plasma was not freely diffusible through a semipermeable membrane presumably due to combination with protein. Since that time other procedures including ultrafiltration, the determination of the solubility of calcium salts, such as $CaCO_3$, in solutions of plasma proteins, and the contraction of the frog heart, have been used as techniques to study the problem. The latter procedure is a means of estimating Ca^{++} concentration directly. If the concentration of ultrafiltrable calcium should exceed the concentration of Ca^{++}, the difference would indicate the amount of calcium in unionized combination with a diffusible or ultrafiltrable anion. When allowance is made for the Donnan equilibrium effect in ultrafiltration experiments, the results indicate that not more than 5 per cent of calcium of the plasma is present in such a non-ionized, diffusible form.

From observations, with the aid of the frog heart, on blood sera of varying calcium and protein concentration, McLean and Hastings (1934) (1935) concluded that the Ca^{++} of serum could, as a first approximation, be expressed by the Mass Law Equation

$$\frac{[\mathrm{Ca}^{++}] \times [\mathrm{Prot}^{=}]}{[\mathrm{CaProt}]} = \mathrm{K} \quad \text{or}$$

$$\mathrm{pCa}^{++} + \mathrm{pProt}^{=} - \mathrm{pCaProt} = \mathrm{pK}_{\mathrm{CaProt}}$$

It is implied in this equation that the protein combines with calcium as if it were present in the form of divalent anions. The value of $\mathrm{pK}_{\mathrm{CaProt}}$ of the total serum proteins, as they exist in normal human serum, was reported to be 2.22 ± 0.07.

Essential confirmation of this value was found by determining the increase of solubility of $CaCO_3$ in solutions of serum proteins (Weir and Hastings, 1936). The value 2.17 ± 0.07 was found in concentrated

solutions of serum proteins by Chu and Hastings (1938). Morison, R. McLean and Jackson (1938) found similar values in the serum of hyperthyroid and myxedema patients.

Greenberg, Larsen and Tufts (1934, 37) have avoided the uncertainty in the assumptions that the proteins act as dibasic acids and that the the calcium-combining power is proportional to the base-combining power as determined from titration curves. They expressed the Mass Law Equation in the form

$$\frac{P}{CaP} = \frac{1}{A} + \frac{B}{Ca_D^{++}}$$

in which A represents the maximum calcium-combining capacity per gram of protein, P, and A $\times$ B = K. Ca_D^{++} represents the diffusible calcium. If this application of the Mass Law is valid, plotting P/CaProt against $\frac{1}{Ca_D^{++}}$ should give a straight line. The term B would be represented by the slope and $\frac{1}{A}$ by the intercept. Greenberg and Tufts (1937) tested the equation for human and dog blood over a fairly wide range of protein concentration, assuming that all the diffusible calcium is ionized. Their results may be described by the equation

$$\frac{P}{CaP} = 0.403 + \frac{5.80}{Ca_D^{++}},$$ which leads to the value, $pK_{CaProt} = 2.44$.

Since total Ca = Ca^{++} + CaProt, and total protein = $Protein^{=}$ + CaProt, the Mass Law relation can be expressed as:

$$\text{Total Ca} = \frac{[Ca^{++}] \times [\text{Total Prot}]}{[Ca^{++}] + K} + Ca^{++}$$

If the value of Ca^{++} is constant, as in normal sera, or as in those with low protein content in the absence of a disturbance of calcium metabolism, the relation between total calcium and total protein can be expressed by the simple regression equation:

$$\text{Total Ca} = \text{m Total Prot} + \text{b}$$

M. Miller (1937) determined the total calcium, albumin, globulin, and total protein in the serum, and the relatively protein-poor pleural or ascitic fluid of twenty-five patients. The total protein of the sera was either low or near the normal value. A statistical analysis of his

results indicated that the relation of calcium to the protein could be expressed by the equations: Δ Ca = 0.561 $\times$ Δ Protein, or Δ Ca = 0.639 $\times$ Δ Albumin + 0.435 $\times$ Δ Globulin. Δ represents the differences between the concentration in the serum and the transudates. The relative calcium-combining powers of albumin and globulin appeared to be proportional to their base-combining powers. Although most of the values obtained for calcium and protein were explainable on the basis of the expressions given above, it may be significant that the values on two patients showed such a deviation that they could not be explained on the same basis.

The simple regression equation, Total Ca = m Total Prot + b, does not hold in cases of lymphogranuloma inguinale, which is characterized by a hyperglobulinemia (Gutman and Gutman, 1936, 1937). The discrepancies suggested that different serum globulins bound different amounts of calcium in these sera. Better agreement between the observed and the calculated values were obtained from the equation:

$$\text{Total Ca} = m_1 \text{ albumin} + m_2 \text{ "globulin II"} + m_3 \text{ globulin I} + b,$$

where b = 5.8 $\pm$ 0.2 mgm. per 100 grams serum H_2O.

m_1 is 0.7 to 0.9 mgm. calcium per gram albumin,

m_2 globulin II is a constant of the order of 1.0 to 1.5 mgm. calcium per 100 grams of serum H_2O.

m_3 is of the order of 0.1 to 0.2 mgm. calcium per gram of globulin I,

globulin I is assumed to represent all the globulin in excess of a total globulin concentration of 3.0 grams per 100 cc.

Jeanneny and Servantie (1937) have also questioned the applicability of the simple equation in hyperglobulinemia. The findings of Roepke and Hughes (1935) are also pertinent to this question. Their results indicate that the very great increase in the calcium of laying chickens (20 mgm./100 cc.) coincides with an increase of the phospholipin and the appearance in the blood stream of a phosphoprotein similar to ovovitellin. Although part of the inorganic phosphate is not ultrafiltrable and, therefore, possibly exists as a precipitate with part of the calcium, a larger portion of the increased calcium may be combined with the phosphoprotein, or possibly with the phospholipin as an undissociated non-diffusible compound.

Several globulins of the blood plasma have been purified by Green, and their calcium-combining properties determined with the aid of the frog heart preparation by Drinker, Green and Hastings (1939). Con-

siderable difference was found among the various globulins in respect to their pK_{CaProt} as follows:

PROTEIN	pK_{CaProt}	CaProt*
		mM per gram
Pseudoglobulin	2.96	0.024
Euglobulin P_I	2.70	0.037
Euglobulin P_{II}	2.00	0.005
Euglobulin P_{III}	3.13	0.086

* Calculated for the condition that Ca^{++} = 1 mM per kilo H_2O.

It is obvious from the results that hyperglobulinemia which involved an increase in one fraction predominantly would result in a variation from the value 2.22 found usually for the pK_{CaProt} of the blood serum. This offers an explanation of the results found by Gutman and Gutman.

The calcium-combining power of the proteins referred to in the experiment above has been determined at pH 7.4. If the equation proposed by McLean and Hastings is valid, one would expect the proteins to combine with more calcium at pHs higher than 7.4, and less calcium at lower pHs. The experiments of Weir and Hastings, over the pH range 7.0 to 7.8 suggest that this is true, although the pH range covered is too small to be conclusive. In this connection, Seekles (1936) has found that the ultrafiltrable calcium of the blood increases about 7 per cent when the pH is changed from 7.4 to 7.0, and decreases about 2 per cent when the pH is increased to 8.0 from 7.4.

The similarity in results obtained by means of solubility experiments and the Straub frog heart on solutions of serum proteins gives support to the validity of the results obtained by the latter method of determining the concentration of calcium ions. Certain precautions in the use of the latter technique are implied in the work of G. Orzechowski (1936) who found that various finely divided substances,—kaolin, barium sulfate, calcium carbonate, or oxalate, increased the amplitude of the beat of the hypodynamic heart. This suggests that caution should be used in the interpretation of results obtained with blood in which a calcium precipitate may be present. Other experiments concerning the influence of Ca^{++} on the isolated heart have been carried out by T. Takezaki (1936), Nahum and Hoft (1937), and Hagen and Singer (1936). Additional procedures which might lead to other means of measuring Ca^{++} concentrations are desirable. In that con-

nection, the determination of the Ca^{++} *concentration in milk* by means of the solubility of the calcium salt of tropeolin 00 (R. Nordbö, 1939) is of interest. The apparent solubility product of $CaSO_4$, $CaCO_3$ and tertiary calcium phosphate in various organic acids and the drop in pH observed when $CaCl_2$ is added to a partially neutralized solution of the acids has been employed by Greenwald (1938) to measure the dissociation of the calcium salts.

Blood inorganic phosphate. The phosphate of the blood is practically completely ultrafiltrable and diffusible when the concentrations of both calcium and phosphate of the blood plasma are near the normal levels. Calcium and phosphate apparently form a small amount of undissociated salt at concentrations lower than that at which precipitation can be initiated, but the amount is so small as to be hardly detectable by the available procedures for the determination of $[Ca^{++}]$. When the concentration of either calcium or phosphate is sufficiently increased in blood plasma, a "colloidal calcium phosphate" complex is formed (Laskowski, 1933; Greenberg, Larsen and Tufts, 1934; McLean and Hinrichs, 1938). Evidence is lacking to indicate whether the precipitate differs from the calcium phosphate precipitate formed in organic solutions at similar concentrations of calcium and phosphate ions. When found in blood, the precipitate is prevented from settling out by virtue of the protective action of the protein colloids.

McLean and Hinrichs (1938) injected phosphate intravenously and determined the Ca^{++} of the blood plasma by means of the frog heart. The difference between the total calcium, and the sum of Ca^{++} (as determined by the frog heart), and the calcium bound by protein (calculated by the procedure of McLean and Hastings) were assumed to represent the amount of colloidal calcium phosphate "complex." The results indicated that the formation of the colloid continues over a period of hours after the blood is withdrawn from the animal, and until the product $Ca^{++} \times P$ of the blood plasma (in mM per liter) approaches 3. Gersh (1938) finds that injected calcium phosphate or that formed in the blood plasma by increasing the calcium concentration is phagocytized in the liver, spleen, and bone marrow.

The dissociation constants of phosphoric acid in solutions of ionic strength similar to that of blood have been determined by Sendroy and Hastings (1926), and can reasonably be expected to be applicable to the calculation of the concentrations of the three phosphate ions, $H_2PO_4^-$, $HPO_4^=$, and $PO_4^{\equiv}$, in blood plasma.

Formation and composition of the bone salt. The earlier literature concerning the composition of the bone salt is to be found in the excellent review of Huggins (1937).

The chemical analyses of inorganic matter of calcified structures have demonstrated that its composition can be expressed approximately as $(Ca_3PO_4)_nCaCO_3$ in which n is between 2 and 3 (Logan, 1935b). Between 3 and 5 per cent of the calcium may be replaced by magnesium, and between 2 and 3 per cent by other bases, chiefly sodium (Gabriel, 1894). Some of the variation in composition to be found in the literature is undoubtedly due to faulty methods of analysis or is a consequence of dry ashing, which was formerly a standard procedure in the preliminary preparation of the material.

Nevertheless, serial analyses have amply demonstrated that the composition is not always the same. The carbonate of the bone increases with age in certain species—for instance, in rats (Kramer and Shear, 1928) and dogs (Gabriel, 1894)—and the relation of calcium to magnesium and carbonate has been found to vary with age in bone (Swanson and Iob, 1937), and is quite different in enamel, dentin, and bone (Armstrong, 1935; Logan, 1935b; Armstrong and Brekhus, 1935). To express accurately the composition of the total inorganic material of different calcified tissues of different species or of one species at different ages by a single chemical formula is therefore out of the question.

The inorganic matter of bone shows the same lines in the x-ray spectrogram as do the apatite minerals, one member of which, dahllite, has a chemical composition similar to that of bone. Calcium phosphate precipitates formed in neutral or alkaline solutions give x-ray spectrographs similar to bone or apatite, whether their chemical composition approximates $Ca_3(PO_4)_2$, or $[Ca_3(PO_4)_2]_2CaCO_3$ (de Jong, 1926; Taylor and Sheard, 1929). The absence of $CaCO_3$ or $CaHPO_4$ as independent substances is indicated by the absence of lines characteristic of any of their crystal forms (Roseberry, Hastings and Morse, 1931). Since the crystals of the bone salts are always small, not larger than 10^{-4} cm. and often between 10^{-5} to 10^{-6} cm., the diffraction lines are somewhat diffuse (de Jong, 1926; Moeller and Trömel, 1933; Bale, Hodge and Warren, 1934; Reynolds et al., 1938). The surface, per unit weight, of these crystals would be relatively much greater than that of crystals sufficiently large to be visible with a microscope. Such submicroscopic crystals would consequently present excellent conditions for adsorption.

In slightly acid solution, the calcium phosphate precipitate formed is $CaHPO_4$, which forms relatively large, regular crystals, and has a diffraction pattern entirely distinct from the bone salt.

Important x-ray studies in relation to the position of the atoms in the molecule of apatite minerals and tooth substance have been made by Gruner, McConnell, and Armstrong (1937), McConnell (1938), and Hodge Le Fevre and Bale (1938). The union of the inorganic salt and the protein of bone has been studied by Caglioti and Giganti (1936), who found that the linear dimension of the elementary cell of the apatite along the *c* axis is the same as that of two peptide linkages ($c_0 = 6.9$ Å). That the crystallites are arranged in a regular pattern accommodating the functional demands on the tissue appears evident, (Caglioti, 1936, Reynolds et al., 1938; Stuhler, 1936). The longitudinal orientation of the crystallites of normal bone is not evident in rachitic bone, nor is it regained on recovery from the deficiency (Clark and Mrgudich, 1934). Fluoride poisoning also disturbs the orientation, and, thereby, unfavorably alters the physical characteristics of the bones and teeth of rats, according to Reynolds et al. (1938). Reynolds, Hayden and Corrigan (1939) found that the diffraction pattern of the inorganic matter of bone in osteitis fibrosa cystica showed a structure different from that of apatite.

Ion products. Contributions to our present understanding of the solubility relations of the bone salt began with the application of the Mass Law to the problem.

At first sight, it would appear that the bone salt and body fluids provide an example of an equilibrium between a slightly soluble salt and a liquid phase to which the solubility product law is applicable. The problem has proved to be much more complicated than this, due to the fact that the calcium salt which is finally present in bone is different from the one which first forms. This means that the product of calcium × phosphate ion concentrations necessary to form the precipitate is greater than the product at which it will dissolve. In considering the question of the biological significance of calcium × phosphate ion products, it is, therefore, necessary to define whether one is concerned with precipitation or solution.

The first demonstration that there is a quantitative relation between the concentrations of calcium and phosphate of blood serum and calcification in vivo was provided by the classic work of Howland and Kramer (1922). They showed that in rickets, the product Total Ca × Total Phosphate (expressed as milligrams per cent) was 35 or less; in normal plasma, the product was 40 or greater. Although the product

of Howland and Kramer can now be more accurately expressed in terms of $[Ca^{++}] \times [HPO_4^{=}]$, the original formulation still retains its practical value. However, this simple relation is not alone adequate to describe the formation of the bone salt.

Holt, LaMer and Chown (1925), and Sendroy and Hastings (1926) equilibrated solutions containing calcium and phosphate, and, in some cases carbonate ions, with relatively large amounts of calcium phosphate precipitates. The ion product, $[Ca^{++}]^3 \times [PO_4^{\equiv}]^2$, calculated from the calcium and phosphate content of the solution and the pH, after prolonged shaking, was 10^{-26} to 10^{-27} for solutions of the same ionic strength as blood plasma. Because the product, $[Ca^{++}]^3 \times [PO_4^{\equiv}]^2$, calculated from the original calcium and phosphate ion concentrations of the blood plasma, is $10^{-23.0}$ to $10^{-23.5}$, the results suggested that the blood plasma was greatly supersaturated with respect to the bone salt. Later, a "solubility product" for bone and dahllite was determined as described above. The product, $[Ca^{++}]^7 \times [PO_4^{\equiv}]^4 \times [CO_3^{=}]$, was thought to represent equilibrium conditions with these solids. The results led to similar conclusions concerning the supersaturation of the blood plasma (Browman, 1935).

Wendt and Clarke (1923) were the first to suggest that the formation of $CaHPO_4$ was a step in the formation of "tertiary calcium phosphate." Shear and Kramer (1928) equilibrated blood plasma with $CaHPO_4$ and concluded that blood plasma was undersaturated with respect to this salt. The importance of this line of attack was subsequently not fully appreciated, because x-ray examination made untenable the hypothesis that substances of composition similar to the bone salt could be mixtures or solid solutions of $CaHPO_4$, $Ca(OH)_2$, and $CaCO_3$.

The concentrations of calcium, phosphate, and carbonate required to initiate precipitation of the inorganic substances, which have the crystal lattice of bone or apatite, depends on the steps involved in their formation. If formed by simultaneous union of all of the ions, then it should dissolve at essentially the same concentrations as those necessary for its formation. The probability of the occurrence of such a simultaneous combination of all the necessary ions is so low, however, that the formation of the final bone salt in one step is highly improbable.

A study of the course of precipitation indicated that the precipitate formed by more than one step (Logan and Taylor, 1937). The following evidence in favor of that viewpoint was obtained by adding calcium chloride to phosphate solutions maintained at pH 7.4, and observing the course of the reaction for as long as 30 days. It was found that:

1. Precipitates formed from solutions containing excess calcium

(but no CO_2), a few minutes after adding the reagents together, change in composition by removing calcium (and presumably OH), but no phosphate, from the solution.

2. The precipitates first formed from solutions containing an excess of phosphate always lose phosphate to the liquid phase.

3. If carbonate is present in the solution, the loss of phosphate from the precipitate parallels the CO_2 uptake.

4. Calcium, carbonate, and hydroxyl ion may be removed from solution, even when their concentrations are such that the ion products $[Ca^{++}] \times [CO_3^{=}]$ and $[Ca^{++}] \times [OH^{-}]^2$ are less than the solubility products of $CaCO_3$ and $Ca(OH)_2$. Other ions forming insoluble calcium salts—i.e., fluoride or alizarin, are taken up in large amounts by the precipitate. (It would reasonably follow, therefore, that Ca^{++} and $HPO_4^{=}$ may be taken up by the precipitate at concentrations below the solubility product of $CaHPO_4$. The composition of precipitates formed from solutions containing phosphate in excess can best be explained on the basis of this assumption.)

5. The composition of the precipitate after long equilibration depends on the composition of the liquid phase. For instance, the amount of CO_2 in the precipitate is increased as the products $[Ca^{++}] \times [CO_3^{=}]$ of the liquid phase approach the solubility product of $CaCO_3$. When the precipitate (such as bone salt) is equilibrated with a solution different in composition from that in which it was formed, the composition of the precipitate changes even though the possibility of recrystallization is precluded. Ions, such as $CO_3^{=}$, can be exchanged for OH^- to bring the precipitate in equilibrium with the new liquid phase (Logan and Taylor, 1938).

Steps in the formation of the precipitate. The steps (as visualized by Logan and Taylor, 1938) concerned with the formation of the bone salt, will now be described, starting with the last step and subsequently describing each immediately preceding one. The last step in the formation of the bone salt is accomplished by the uptake of $CO_3^{=}$, (OH^-), etc., together with calcium.[3] A preceding step must be the formation of the crystal lattice of the final product with the apatite crystal structure, possessing the forces and providing the spaces necessary to accomplish the last step. Its chemical composition is presumably $Ca_3(PO_4)_2 \times H_2O$, or a ratio of 3 Ca to 2 PO_4.

[3] The precipitate can exchange CO_3, OH, and PO_4 (or HPO_4) as the composition of the liquid phase changes. Consequently, Ca and CO_3 need not be taken up or removed in equivalent quantities. The same applies to gain and loss of Ca and HPO_4.

Loss of phosphate from the precipitate during the course of precipitation is evidence, however, that this is not the first step. The first step is probably an aggregation of calcium and phosphate ions in the ratio of 1 to 1. Because precipitates formed in the physiological range of pH have the crystal lattice of the final substance almost immediately after their formation, it appears that the first aggregation does not attain the size and orientation necessary for crystal formation in this ratio, but loses, under these conditions, phosphate ions with the formation of the apatite crystal lattice. Growth of the crystals is then to be regarded as a repetition of the process—namely, removal of calcium and phosphate by the lattice structure. Regardless of its composition in other respects, during the early formation of the precipitate, the solution is essentially saturated in respect to $CaHPO_4$. The precipitate should, therefore, attain its maximum content of phosphate early in the course of precipitation, and subsequently lose it as a consequence of the succeeding reactions. (See footnote 2.)

If the precipitate forms according to these steps, it follows that the concentrations of calcium and phosphate ions necessary to form the precipitate are greater than those at which the precipitate will dissolve.

The ion product, $[Ca^{++}]^3 \times [PO_4^{\equiv}]^2$, at equilibrium should increase as the amount of solid equilibrated with a given amount of solution is decreased. The ion product nearest to that representing the concentrations required for formation of the precipitate should be that obtained with a minimum amount of solid. Equilibration of various calcium phosphate precipitates, including the bone salt, with inorganic solutions and blood plasma (Logan and Taylor, 1937; Logan and Kane, 1939), showed that the ion product, $[Ca^{++}]^3 \times [PO_4^{\equiv}]^2$, obtained with large amounts of solid, was the same as that previously found by others for similar amounts, but when the amount was decreased, the value approached 10^{-23}. This is essentially the value of the ion product, $[Ca^{++}]^3 \times [PO_4^{\equiv}]^2$, calculated from the $[Ca^{++}]$, the phosphate and the pH of normal blood plasma. When calculated as $[Ca^{++}] \times [HPO_4^{=}]$ for solutions maintained between pH 7.0 and 7.6, the values approached $10^{-5.6}$, as the solid was decreased in amount. This is essentially the value determined for the solubility product of $CaHPO_4$ (Shear and Kramer, 1928). It may, therefore, be the ion product $[Ca^{++}] \times [HPO_4^{=}]$ which is the deciding factor in the initiation of precipitation even though $CaHPO_4$ as a crystal entity is not produced under these conditions.

The results, therefore, indicate that normal blood plasma is not super-

saturated with respect to the bone salt, in the sense that it could form spontaneously at the concentration of calcium and phosphate present. Once having been formed, the bone salt could not dissolve at the concentrations of calcium and phosphate present in the blood plasma. The intermediation of cells at the site of deposition, therefore, is necessary for the initiation of the precipitation or for the subsequent solution, such as takes place in the relocation of the bone salt during the process of growth. Therefore, in the following discussion, the contributions of the last few years will, as far as possible, be considered from the standpoint of the influence of various factors: 1, on absorption of calcium and phosphorus; 2, on the activity of the bone cells, and 3, on urinary excretion of calcium and phosphorus.

Absorption. The net amount of calcium absorbed from the intestinal tract each day is the difference between the total amount which is absorbed and that which enters the lumen of the intestine as a constituent of the intestinal juices. Investigations of the calcium content of the various digestive juices have been reviewed by Nicolaysen (1934). In general, the values for the calcium content of saliva, liver bile, and succus entericus approximate those of normal blood plasma. Those for gastric juice and pancreatic juice are nearer the values for the ultrafiltrable calcium of the plasma. Four to ten liters of digestive juices are secreted into the lumen of the intestines per day by a normal man (Rowntree, 1922; Adolph, 1933). Consequently, 0.3 to 0.8 gram of calcium are secreted into the intestinal tract per day. The total amount absorbed by the intestinal tract may, therefore, amount to more than twice that which is secreted in the urine plus that which is deposited in the bone per day. If the cells of the intestinal wall have a diminished ability to absorb calcium, or if conditions in the lumen of the intestine are such that the calcium is precipitated (e.g., as calcium phosphate) before absorption, the net amount absorbed might appear as a negative quantity. There is thus provided an explanation for the loss of previously absorbed calcium (or phosphate).

At various times, an active excretion of calcium into the colon has been postulated. The results, however, have been based on experiments which are open to some question. For instance, Cowell (1937) analyzed fecal pellets taken from the cecum, sacculated colon, thick walled colon, and anus of rabbits. He found that the calcium content of the dried pellets increased as fecal matter descended in the sacculated and thick walled colon. The exterior of the pellets contained more calcium than did the interior. From these facts, he was inclined to consider

the possibility that excretion of calcium into the colon occurred in rabbits. The addition of calcium to the fecal mass from this source was, however, small compared with the urinary excretion which occurred on the same diet. Furthermore, it is not clear from his experiments whether or not intestinal juices containing calcium enter the lumen of the rabbits' intestine at this point just as they do at higher levels in other species without any reference to the animals' need for calcium. It is significant that the calcium of the feces was not increased when the blood calcium was raised to 20 mgm. per 100 cc.

Calcium (or magnesium) when injected intravenously as the gluconate into normal well-nourished humans, is excreted in the urine rather than in the feces according to McCance and Widdowson (1939).

Availability of calcium from various sources. Mellanby (1922) first called attention to the so-called anti-calcifying properties of cereals. Because 50 to 80 per cent of the phosphate in cereals occurs as phytin phosphorus (inositol hexaphosphoric acid ester) which is hydrolyzed with difficulty and forms a difficultly soluble calcium salt, it is probable that the phosphate of cereals is not well absorbed. Bruce and Callow (1934), McCance and Widdowson (1935), and Lowe and Steenbock (1936) consider that the anti-calcifying properties of a cereal diet result from deficiency of available phosphate. In the absence of calcium salts, the intestinal bacteria may hydrolyze a small amount of the phytin and thus favor its utilization. When excess $CaCO_3$ is present, the organic phosphate is presumably precipitated before being hydrolyzed, and, consequently, is rendered unavailable for absorption. On the other hand, Palmer and Mottram (1937) find that the tendency of cereals to produce rickets resides in their low calcium and high phosphate content, and can be counteracted by adding calcium lactate. Additional phytin, instead of increasing the rachitic properties of the diet, reduced it. Rats grew to maturity on white flour plus wheat germ and $CaCO_3$ added in an amount sufficient to make the Ca/P ratio 2/1 (Palmer, 1939).

From 36 to 63 per cent of phytin fed to humans is excreted unchanged (McCance and Widdowson, 1935). Patwardhan (1937) found an enzyme in the intestine of rats which splits sodium phytate. Lack of vitamin D probably does not decrease the phosphatases of the intestinal tract, however, because Nicolaysen (1937) found that glycerophosphoric acid and casein phosphate were utilized as well by rachitic as by normal animals.

McDougall (1938) found that the addition of 11 per cent of lard

or cocoanut oil prevented rickets in rats fed a diet containing 75 per cent cereal. She considers that the fat helps to acidify the intestinal tract and improves calcium absorption by the formation of a soluble bile salt-calcium soap complex. Lowe and Steenbock (1936) noted no beneficial effects from the addition of similar amounts of lard or lactose to the phytin-containing diets.

That oxalic acid in spinach prevents absorption of calcium has again been demonstrated by Brull and Barac (1938), and also by Fairbanks and Mitchell (1938). Mackenzie and McCollum (1937) had previously noted that when the calcium content of the diet was limited, oxalate retarded growth. When the diet contained adequate amounts of calcium and vitamin D, the oxalate decreased the percentage of the ash of the bone, one mol of oxalate evidently preventing the absorption of less than one mol of calcium. Herkel and Koch (1936) found that calcium interferes with the absorption of oxalate, while HCl and histamine promoted its absorption.

Adolph, Wang and Smith (1938) added 25 per cent of regenerated cellulose to the diet of rats and studied the excretion of calcium. The results indicated that the extra roughage did not increase the fecal loss of calcium. On the other hand, Westerlund (1938) found that 20 per cent of paper pulp in the food caused a moderate increase in intestinal excretion of calcium, and that rats could be maintained on a smaller supply of calcium in the absence of the cellulose. Oat straw increased the fecal calcium excretion of rats when it comprised more than 20 per cent of the dry weight of the food (Westerlund, 1939).

Kao, Cormer and Sherman (1938) compared the availability of the calcium in milk and Chinese cabbage. Control rats obtained the calcium entirely from milk in the diet. In the experimental animals, half of the calcium was supplied from cabbage and half from milk. As judged by the calcium content of the bodies of the rats after 60 days on the diets, the calcium of the cabbage appeared to be utilized almost as well as that from the milk.

Lactose favors absorption of calcium, presumably by virtue of the fact that, on the one hand, it forms a small amount of undissociated soluble calcium salt, and, on the other, fermentation of the lactose results in lactic acid which permits absorption by acidification of the intestinal contents (Robinson and Duncan, 1931). Precipitation of calcium phosphate in the intestine is, thereby, retarded. However, lactose is not as effective in facilitating absorption as cod-liver oil, according to Outhouse, Smith and Twomey (1938). Comparison of

sucrose, fructose and lactose, when they constituted 60 to 70 per cent of the diet, indicated that the digestibility of organic constituents was decreased, but the metabolic utilization of calcium was definitely increased by the above sugars as compared with glucose. The rats fed lactose grew at a rate which was 40 per cent less than that of the glucose-fed controls. Nevertheless, they deposited 97 per cent as much calcium and 94 per cent as much phosphate as the controls (Mitchell, Hamilton and Beadles, 1937).

French and Cowgill (1937) carried out balance experiments on two young dogs and one adult dog. The results indicated that lactose in the diet favored calcium utilization only in the immature animals. Experiments on the effect of lactose on absorption by rats were also attempted by the Bergeim technique, but these gave doubtful results.

Shohl (1937) found that the addition of a citric acid:sodium citrate mixture to a diet which produced mild rickets in rats converted this into one which was non-rachitic. The mixture appeared to be more anti-rachitic than equivalent amounts of citric acid or sodium citrate alone, or mixtures of acetic, lactic, malonic or succinic acid and their salts. The beneficial effects were, therefore, thought to result from a combination of factors. Precipitation of calcium phosphate in the intestinal tract is retarded because the pH of the intestinal contents is lowered, and because the slightly dissociated, and more soluble, calcium citrate is formed. The deposition of calcium phosphate after absorption is favored because the pH of the tissue fluids is increased. NH_4Cl:$(NH_4)_2CO_3$ mixtures, as might be expected, produced the opposite result—namely, converted borderline diets into rachitic diets. This mixture tended to make the intestinal contents relatively more alkaline and, thereby, favored precipitation of calcium phosphate before absorption, and by lowering the pH of the tissues, tended to retard precipitation after absorption.

That citrate added to the rachitogenic diet increases the per cent of ash in the bone has been shown by Hathaway and Meyer (1939). Potassium citrate was more effective than the sodium salt. Lanford (1939) found that rats grew faster and stored a slightly larger per cent of the calcium when the diet, composed of a wheat and milk mixture, was supplemented by 5 cc. of orange juice per day.

In the absence of vitamin D, Shohl and Wolbach (1935) found that production of rickets in rats appears to be a function of both the ratio of calcium to phosphate in the food and the absolute amount of each. Theiler, DuToit and Malan (1937) report that an intake of 0.8 gram

phosphate was insufficient to prevent rickets in pigs when the calcium/phosphate ratio was 7.5/1. When the calcium/phosphate ratio was 1/10, 1 gram of calcium per day was insufficient to prevent osteoporosis. Jones (1939) produced rickets in rats on a diet consisting entirely of purified materials. Vitamin D and the calcium to phosphate ratio of the food appear to be the only factors involved. Severe rickets were obtained when diets contained 3 per cent $CaCO_3$ and not more than 0.1 per cent available phosphate.

That rickets can be produced in rats by feeding substances, other than calcium, which precipitate an insoluble phosphate in the intestinal tract has been recognized for several years. Investigation of this type of deficiency has been continued by Jones (1938). He gave 5000 International Units of irradiated ergosterol to rats fed the Steenbock diet 502 (high calcium, low phosphate), supplemented with yeast and 1 per cent beryllium carbonate. Controls fed the same diet, but receiving no irradiated ergosterol developed very severe rickets. The blood inorganic phosphate dropped from 9 mgm. per 100 cc. to less than 3 mgm. per 100 cc. Addition of the irradiated ergosterol increased the inorganic phosphate of the serum slightly and improved somewhat the ash content of the femurs. The toxicity of large doses of irradiated ergosterol was not reduced by adding phosphate to the diet. A few of the rats, which had been made severely rachitic by the addition of aluminum to the high calcium-low phosphate diet developed tetany when the aluminum was removed. The tetany was coincident with an increase of the blood inorganic phosphate.

Blumberg, Shelling and Jackson (1938) produced rickets in rats by substituting $MnCl_2$ for calcium in a high calcium-low phosphate diet, and by adding the same amount of $MnCl_2$ to a diet containing optimum amounts of calcium and phosphate. In the latter case, vitamin D protected the animals against rickets. The soluble manganese salt interfered somewhat with growth. A small amount of manganese in the diet (100 parts per million) is necessary for the normal development of the bones of chickens (Caskey, Gallup and Norris, 1939). The minimum amount necessary varies with the amount of calcium phosphate precipitated in the intestinal tract (Wilgus and Patton, 1939), because the latter caries down manganese thereby decreasing its availability.

Darby and Mallon (1937) found that chlorophyll did not improve the calcium retention of young women on a diet which contained whole milk. Diminished calcium absorption and tetany were observed in humans by Hetenyl (1938) accompanying chronic catarrh of the small

intestine. Brull and Barac (1938) call attention to the loss of calcium as soaps in the feces and consequent decalcification of the bones, as a result of the steatorrhea of sprue. Similar observations were made in idiopathic steatorrhea (Bassett et al., 1939a). Loss of vitamin D with fatty acids in the feces may be a factor contributing to the deficient absorption of calcium (Bassett et al., 1939b). It has also been observed by Bussabarger, Freeman and Ivy (1938) that gastrectomy causes osteoporosis in puppies. Decreased calcium absorption, as a consequence of increased speed of intestinal transport following the operation, may be largely responsible for the inadequate calcification.

Influence of growth and calcium reserves on absorption. Parathyroid oversecretion has no influence on calcium absorption or excretion into the intestinal tract, as is shown by the experiments of Aub, Tibbets and McLean (1937). However, they observed that resumption of growth, following removal of a parathyroid tumor, favorably influences calcium absorption. The unknown factor responsible for this change is not related to vitamin D. Further knowledge of the influence of growth "per se" on intestinal absorption would appear to be desirable.

Absorption from the intestinal tract may be influenced by the calcium reserves in the animals' tissues, at the beginning of the test periods, if one may judge from experiments on rats by Rottensten (1938). In a preliminary period of 4 weeks, he fed 6 rats on a diet which contained 0.4 per cent phosphate and 0.15 per cent calcium. A group of litter mates were fed the same amounts of a diet which contained 0.4 per cent phosphate and 0.8 per cent calcium. All the rats were then put on a diet containing 0.4 per cent calcium. Paired litter mates received equal amounts of food per day. The rats which had previously been on the low calcium diet stored an average of 924 mgm. calcium and 580 mgm. phosphate in 35 days as compared with 600 mgm. calcium and 471 mgm. phosphate, stored by the rats which had previously been on the high calcium diets. The better retention of calcium and phosphorus by the depleted rats results from increased absorption from the intestinal tract, not from decreased excretion in the urine. Individual differences in the ability of pullets to absorb and utilize calcium was noted by Morgan and Mitchell (1938). Hens showing high egg production also were able to absorb and utilize large amounts of calcium.

Effect of vitamin D on absorption. Nicolaysen (1937) compared the absorption of calcium by rats made rachitic on the diet of Steenbock and Black with that of rats fed on the same diet but in which rickets was prevented by the addition of cod-liver oil. During the period used

for testing absorption, the rats were fed on a diet which was essentially devoid of phosphate, but contained varying amounts of calcium and vice-versa,—i.e., essentially devoid of calcium but containing varying amounts of phosphate. The results showed that, in the absence of calcium, the presence of rickets did not interfere with phosphate absorption, because the phosphate was absorbed practically completely whether administered as inorganic phosphate, glycerophosphate, or casein. When the maximum amount was administered (15 mgm. phosphate per day), tetany sometimes developed.

When 15 mgm. of calcium per day were administered (and only 1.1 mgm. phosphate), the calcium was absorbed completely by normal rats. The rachitic rats, on the same intake, absorbed only 5.4 mgm. Although greater amounts of calcium in the diet of the rachitic rats resulted in increased absorption of calcium, the per cent of ingested calcium which was absorbed was decreased. The disparity between the absorption of calcium by normal and rachitic rats at levels of 90 and 180 mgm. per day was 19 and 47 mgm. respectively, indicating quite clearly that vitamin D favorably influences calcium absorption, but is without effect on phosphate absorption. Absorption of calcium by the deficient rats was less than that of normal rats, but it was more than that which would be deposited per day by normally nourished rats. These results indicate that the essential defect in rickets is decreased ability to absorb a sufficient amount of both calcium and phosphate when they are both present in adequate amounts in the intestinal tract.

Similar evidence that vitamin D increases calcium rather than phosphate absorption is provided by balance experiments on patients with a form of rickets which is very resistant to vitamin D therapy (Albright and Sulkowitch, 1938). Administration of large doses of vitamin D decreased fecal excretion of both calcium and phosphate, and increased the urinary calcium excretion. It was evident from their experiments that calcium given by mouth to untreated patients increased markedly fecal phosphate excretion, but phosphate by mouth increased fecal calcium excretion only slightly. Intravenous administration of calcium or phosphate caused no change in the fecal excretion of these substances. The results are consistent, therefore, with other findings which indicate that re-excretion into the gastro-intestinal tract depends more on the amount of intestinal juices than on the amounts of calcium and phosphate in the blood stream. Vitamin D also increases the absorption of lead if rats are on a rickets-producing diet (Soberl et al., 1938a, b).

Whether or not vitamin D acts directly on the intestinal cells is not

known. This problem has been attacked by Heymann (1937) who found that vitamin D, administered by intramuscular injection, is slowly excreted through the intestinal wall and, in part, reabsorbed with the aid of the bile.

Species differences in absorption and retention. The interpretation of results found in the study of the calcium metabolism of different species must be considered in the light of certain fundamental differences which exist in absorption and excretion by different species. For example, balance experiments on normal, growing rats and dogs ingesting sufficient calcium and phosphate to provide for satisfactory calcification (Henry and Kon, 1939; Kinsman et al., 1939; Shohl and Bennett, 1928), show that they can absorb and utilize 80 to 95 per cent of the intake. However, most balance experiments on humans indicate that absorption usually does not exceed 50 per cent of the intake (Kinsman et al., 1939; Findlay, Paton and Sharpe, 1920; Coons and Blunt, 1930; Sherman and Hawley, 1922). In rats, if the phosphate intake is below the requirement and the calcium greatly in excess, the calcium is largely excreted in the urine (Henry and Kon, 1939; Day and McCollum, 1939). Comparable experiments on humans are not available. Those having a bearing on this point in humans are complicated by a large phosphate intake or a variation in the amount of acid ingested with different amounts of calcium. When allowance is made for these factors (both of which influence the calcium excretion in the urine), it appears that excess calcium intake has but little influence in man on calcium excretion by the kidneys (Haldane, Hill and Luck, 1923; Lieberman, 1931; Orr, Holt, Wilkins and Boone, 1924; Telfer, 1922). The calcium in the urine rarely exceeds 20 per cent of the total calcium intake when the latter is large. Dogs appear to absorb only as much as they utilize (French and Cowgill, 1937; Shohl and Bennett, 1928; Greenwald and Gross, 1929; Givens and Mendel, 1917). The urinary excretion is not over 10 per cent and is often less than 5 per cent of the total when the intake is large (Givens, 1918; Nicolaysen, 1934).

Phosphate, even in normal rats, tends to be absorbed to a greater extent than calcium. The phosphate is not retained unless calcium is absorbed simultaneously. Tisdall and Drake (1938) found that the addition of calcium salts to a low calcium diet increased the retention of phosphate by rats. Excessive intake of phosphate not only decreases the amount of calcium absorbed, but the intake of rats can be increased to the point where it becomes a question of the ability of the kidneys to excrete such large amounts (MacKay and Oliver, 1935; Haldi, Bach-

mann, Wynn and Ensor, 1939). This, in turn, may influence the urinary excretion of calcium secondarily.

Relation of acid-base balance to calcium excretion. That the acidity of the food has a slightly favorable effect on absorption has been noted above. Whether or not acid produced in the course of metabolism has an influence on the calcium and phosphate balance depends on the species used for the experiments. For instance, administration of acid to cats (Logan, 1935) or dogs (Greenwald, 1929; Givens and Mendel, 1917) does not increase fecal or urinary calcium excretion significantly. In rats (Shohl et al., 1932) and in humans, the effect of acid is appreciable; but on ordinary diets, it is a minor factor as compared with others of dietary origin. In fasting humans, ketosis is accompanied by increased excretion of calcium in the urine (Benedict, 1915; Gamble, Ross and Tisdall, 1923). Increased urinary calcium excretion after NH_4Cl administration in humans may be equivalent to 3 to 10 per cent of the extra acid excreted (Loeb et al., 1932; Dennig, Dill and Talbot, 1929; Farquharson et al., 1931). It has also been observed that rabbits, (which cannot produce ammonia to neutralize excreted acid), excrete large amounts of calcium in response to acid administration (Logan, 1935a). These facts probably account for the wide fluctuations noted in the blood calcium and in the composition of the bone of rabbits, making them unsuitable for most investigations on calcium metabolism.

Maintenance requirements of calcium and phosphate. Balance experiments will now be described which were designed to establish the daily requirements of calcium and phosphate. The subjects were considered to be normal or to be suffering only from a deficiency of vitamin D.

From the previous discussion, it follows that in normal humans or those suffering from vitamin D deficiency, differences in calcium retention are primarily related to differences in absorption of calcium from the intestinal tract. (The influence of endocrine dysfunction is considered later.) In rats, the retention is complicated by the fact that not only phosphate but also calcium can be absorbed from the intestinal tract in amounts which are greatly in excess of the amount utilized.

Calcium and phosphate requirements of humans. Outhouse et al. (1939) found that girls 3 to 6 years old, whose diet for 15 weeks had contained 1.8 grams of calcium per day (some of it in the form of $CaHPO_4$), subsequently showed a maximum retention of calcium, on a calcium intake of 0.615 gram per day (supplied as milk solids). Boys required a somewhat higher intake than girls to bring about maximum retention according to Kinsman et al. (1939). About 20 per cent of the calcium of the milk ingested was utilized under these conditions.

On identical diets, the amounts of calcium absorbed and retained by different individuals varies considerably and may not be constant in successive metabolic periods. For instance, Hunscher, Hummel and Macy (1936) made observations for 30 to 60 days on 4 healthy children aged 5 years. On identical diets containing 1 gram of calcium per day, the retentions varied between 0.27 and 0.48 gram of calcium per day. The balances during successive 6 day periods on the same child varied from 27 to 48 per cent. Likewise, Kung and Yeh (1937) found that in balance experiments on 5 women students, 21 to 23 years old, the retention on identical diets varied between 0.007 and 0.082 gram calcium per day. These subjects each received 0.42 gram calcium and 0.97 gram phosphorus per day in the diet, and the urinary calcium of the different individuals varied from 0.031 to 0.232 gram calcium per day. The amount of urinary calcium excretion appeared not to be related to the amount of calcium retained.

Kunerth and Pittman (1939a, b) noted considerable variation in the retention of calcium, phosphorus and nitrogen by normal women in successive 3-day periods. Their results indicated that the calcium, phosphate and nitrogen balances were more favorable on a moderate protein intake than on a low protein intake.

Huff and Pyle (1937) conducted balance experiments on pregnant women who ingested food containing 1 to 2 grams each of calcium and phosphorus per day. The level of ingestion of calcium by these subjects apparently had little influence on the amount of calcium retained. The phosphate ingestion, on the other hand, did influence the amount of calcium and phosphate retained. The subjects in negative calcium balance excreted more calcium in the feces than did those showing a positive balance. The difference in the urinary calcium excretion of the two groups was not significant.

Balance experiments on adult humans indicate that considerable amounts of calcium and phosphate are required for maintenance throughout life. For instance, Owen (1939) found that 520 mgm. calcium and 1200 mgm. phosphorus per day were required to maintain the calcium balance in subjects that ranged from 40 to 69 years of age. L. Brull (1936) found the mean calcium and phosphorus requirements for adults to be 8.2 and 13.9 mgm. per kgm. per day, respectively. Steggerda and Mitchell (1939) found that 9.2 mgm. of calcium per kilo of body weight per day were required to keep an adult in equilibrium. Calcium gluconate or milk solids, when constituting 75 per cent of the calcium intake, appeared to be equally well utilized.

Balance experiments designed to establish better the normal daily

requirement of calcium have also been conducted on young women by M. M. Kramer and I. Gillum (1938) and by M. L. Maxwell (1938); on children by V. A. Porokava (1937), and on students and others by Radsma, Klerks, and J. W. R. Everse (1937).

The essential cause of osteomalacia (as of rickets) is lack of ability to absorb calcium from the intestinal tract according to the balance experiments of Hannon, et al. (1934). That vitamin D is more efficacious than a high calcium diet in improving the calcium balance is also indicated by the balance experiments of Liu et al. (1937) on lactating Chinese women who showed evidence of osteomalacia.

Calcium and phosphate requirements of rats. The experiments of Lanford and Sherman (1938) indicate that when the ratio of calcium and phosphate fed is near the optimum and the amount of each is adequate to support normal nutrition through many generations of rats, the rate of calcification still may not be at its maximum. Increasing the calcium of the diet from 0.2 per cent to 0.8 per cent of the dry mixture caused the offspring of the rats on the higher intakes to attain in one month the percentage of body calcium achieved on the lower level in 5 to 6 months. In this connection, it is interesting to note that H. C. Olsen (1938) finds that mice transfer 11.6 per cent of their calcium reserve to the fetus during pregnancy, and an amount 30 per cent larger than their total reserve during lactation.

H. B. Vickery (1936) has investigated the inorganic salt requirement of a colony of rats which showed rapid gain in weight (5 to 6 grams per day) on the diet employed. The ratio of calcium/phosphate in the food was varied from 1/2 to 1/0.45. When the rats attained a weight of 200 grams, the proportion of ash of the extracted femurs was found to be essentially constant (58 to 60 per cent).

Mendel, Hubbell and Wakeman (1937) compared the effect of several commonly used salt mixtures on the growth rate of rats and the ash content of the femurs. It was shown that if adequate calcium was supplied, many other constituents could be furnished in amounts less than those ordinarily used. The Osborne and Mendel salt mixture was modified to contain about twice the amount of calcium originally recommended. When this mixture constituted 2 per cent of the diet, it supplied an average daily intake of 50 mgm. calcium and 35 mgm. phosphorus, and the rats grew from 60 grams to 200 grams at an average rate of 5 grams per day. The ash content of the femurs at this time (60 per cent) indicated that satisfactory calcification had been obtained.

E. C. Robertson (1937, 1938) finds that a low calcium diet causes

constipation in rats and in children. The appetite of rats appears to be diminished on a low calcium intake. Rottensten (1938) noted restricted food intake on a diet which contained 0.15 per cent calcium. Toepfer and Sherman (1936) noted that adding $CaCO_3$ to a stock diet consisting essentially of 80 per cent whole wheat and 17 per cent milk powder, increased the rate of growth when the rats were permitted unlimited amounts of the diet. The added $CaCO_3$ increased the calcium content of the diet from 0.2 to 0.64 per cent, and made the calcium/phosphate ratio 1.5. On the lower calcium intake, addition of protein as casein increased the rate of growth and improved the calcification. Eppright and Smith (1937) found that with food intake of rats limited to 50 per cent of the usual consumption, addition of calcium and phosphate improved the rate of growth. M. J. L. Dois (1936) found that a diet containing 1 per cent phosphorus and a calcium/phosphate ratio of 3 prevented rickets in chickens over a period of 5 weeks. Couch, Fraps and Sherwood (1937) found that the quantity of vitamin D required for chickens decreased when the calcium content of a diet containing 0.76 to 0.81 per cent phosphorus was increased to 1.71 per cent, or a ratio of calcium/phosphate = 2.2.

SUMMARY. 1. The amount of calcium circulated daily from the blood stream into the intestinal lumen and back again may be as large as the net amount of calcium absorbed from the ingested food.

2. The availability of ingested calcium or phosphate is decreased by substances which tend to precipitate one or the other, or both, in the intestinal tract and favored by those that tend to keep them in solution.

3. Vitamin D primarily favors calcium absorption. Phosphate absorption is increased secondarily by vitamin D as a consequence of greater calcium absorption. Growth and the calcium reserve of the individual may influence calcium absorption.

4. The proportion of the total ingested calcium which is absorbed varies in different species. Differences also exist between species in respect to the relative amounts of the absorbed calcium which is utilized. Acidity of the diet may influence the retention, depending on the species.

Effect of vitamin D on bone cells. That vitamin D may accelerate bone formation by direct action on bone cells has been inferred from the occasional occurrence of low blood calcium and tetany during the treatment of rickets with vitamin D. Recently, Schneider and Steenbock (1939) found that vitamin D added to a rachitic diet deficient in phosphate retarded the growth of rats. The disappearance of the rachitic metaphysis was accompanied by an increase of blood inorganic phos-

phate, indicating that the resumption of bone deposition at the expense of the phosphate of the tissues was associated with the cessation of tissue growth. The uptake of radioactive phosphorus by the bones of rats was examined 64 to 80 hours after administration (as Na_2HPO_4) intraperitoneally and by stomach tube (Cohn and Greenberg, 1939). In both cases, vitamin D increased the deposition of the phosphate in the skeleton 25 to 50 per cent.

Venar and Todd (1936) found that aqueous extracts of viosterol increased the in vitro calcification of fragments of the "growing area" of the tibias of rachitic rats. The amount of calcification varied with the amount of calcium glycerophosphate in the medium, but the smallest amount of vitamin D employed produced as much calcification as larger amounts.

Calciferol (vitamin D_2): Effect of large doses. The conclusions to be drawn from the administration of large amounts of vitamin D raises the question as to whether the "toxic" dose causes changes which are simply exaggerations of those produced by therapeutic doses or whether the material administered may contain more than one physiologically active substance. The latter applies particularly to earlier preparations of irradiated ergosterol, which may have contained the product of excessive irradiation (toxisterol). The subject has been reviewed by Bills (1939) and Park (1939). The therapeutic irradiation product of ergosterol (calciferol) in doses of 100,000 units or more daily raises the blood calcium. The action appears to result from abnormally increased intestinal absorption (Bauer, Marble and Claflin, 1932). Massive doses (460,000 International Units) of crystalline calciferol evidently accelerates the absorption of calcium from the intestinal tract of rats to an abnormal degree (Tweedy et al., 1939). This effect was observed even though the rats were previously thyroparathyroidectomized and nephrectomized. Morgan, Kimmel and Hawkins (1937) compared the action of crystalline calciferol and fish liver concentrates.

Using crystalline vitamin D_2, Kertskin (1938) found that the blood calcium was raised in parathyroid tetany if adequate amounts of calcium were fed. Increase of the blood calcium and changes in the arterioles, especially of the kidney, resulted when 600 γ of vitamin D_2 were administered to thyroparathyroidectomized dogs (Handovsky and Goormaghtigh, 1937).

Dihydrotachysterol. One of the intermediary irradiation products of ergosterol on reduction gives a substance, dihydrotachysterol (A.T. 10) which increases the blood calcium of thyroparathyroidectomized

animals and is used therapeutically for the relief of tetany. The action of the substance appears to be qualitatively similar to that of vitamin D, in that it increases the absorption of calcium and the urinary excretion of phosphate. As compared with vitamin D, the urinary phosphate secretion is increased to a greater extent than is the absorption of calcium. Therefore, no improvement of the calcium balance or cure of rickets results from its administration (Albright, Bloomberg, Drake and Sulkowitch, 1938; Shohl, Fan and Faber, 1939).

Vitamin C. In agreement with the earlier work of Howe (1920, 1921, 1923) and others, Herman, Kramer and Kirgis (1939) found that lack of vitamin C caused resorption of bone of guinea pigs at the base of the cheek teeth and along the edge of the alveolar area. This occurs in adult as well as in growing guinea pigs.

Extensive histological studies of vitamin C deficiency and its relation to calcification have been made (Wolbach and Howe, 1926). This phase of the subject has recently been reviewed by Dalldorf (1939). Advances in the relation of vitamin C to calcium metabolism have perhaps been retarded by the fact that, of the easily available experimental subjects, only guinea pigs and humans are susceptible to the deficiency. The subject merits further consideration because the primary disturbance characterizing this deficiency is observed in the formation of the organic intracellular matrix. Defective calcification of the bones and teeth (which probably are accompanied by negative calcium and phosphate balances) apparently results from inadequate formation of the supporting collagen rather than from a primary disturbance of the absorption or deposition of the inorganic salts.

Effect of parathyroid hormone. The blood calcium of young dogs and humans is much more easily affected by the administration of parathyroid hormone (made from bovine glands) than that of other species, or even of adult dogs. The most striking change following the administration of a potent extract of parathyroids to young dogs is an increased excretion of phosphate in the urine (Greenwald and Gross, 1925; Greenwald, 1926; Albright and Ellsworth, 1929; Albright, Bauer, Cockrill and Ellsworth, 1931; Albright, Bauer and Aub, 1931), accompanied by a decrease of the inorganic phosphate of the blood plasma. The change occurs during the first hour of administration which indicates that the hormone stimulates the kidneys specifically to increased excretion of phosphate.

The hormone apparently also causes active solution of bone. The blood calcium reaches its maximum value 12 to 15 hours after adminis-

tration of the hormone. The increase of blood calcium starts, however, in the first hour after the administration of a fairly large dose. The urinary phosphate excretion continues at a rapid rate until the blood calcium rises to a high level. Subsequently, the urinary phosphate excretion decreases, the blood inorganic phosphate increases far above the normal level, and other evidence of kidney damage appears. The urinary calcium excretion, although increased several hundred per cent, is still quantitatively insignificant (Logan, 1939). The calcium and phosphate thus accumulated in the blood precipitates in some tissues, especially the kidneys (Morgan and Samisch, 1935). That the renal tissue causes additional local concentration in the process of excreting the calcium and phosphate is likely. Primary hyperthyroidism often leads to renal calcinosis (Albright, Baird, Cope and Bloomberg, 1934). The histological changes in the kidneys, resulting from parathyroid administration, have been described by Chown, Lee and Teal (1937).

Parathyroid hormone administered to nephrectomized dogs or rats (Tweedy, Templeton and McJunken, 1936; Tweedy and McNamara, 1936) causes little or no increase of blood calcium. However, nephrectomy alone causes a very rapid increase of the inorganic phosphate of the blood and tissues (Tweedy, Templeton and McJunken, 1936) and, under these conditions, an increase of the blood calcium could hardly be expected.

Shelling, Kajdi and Guth (1938) administered parathyroid hormone to fasting adult dogs in successive doses of 3.3 to 7.5 units per kilo at 4-hour intervals, for 12 hours. In 5 to 20 hours, there resulted a diuresis accompanied by an increase of NaCl excretion. This was followed by anuria and death of the animals. Other dogs were given salt solution intravenously in addition to the parathyroid hormone. These recovered from the treatment. The beneficial effects of the salt administration led them to suggest that dehydration plays an important part in the production of the symptom complex.

It is interesting to note that in several of their experiments, the urinary excretion of phosphate and nitrogen had started to decrease before the diuresis and salt excretion had reached its maximum. This sequence of events corresponds with what was regularly observed (Logan, 1939) following the administration of one large dose of parathyroid hormone to dogs, and leads to the consideration that the increase of phosphate excretion following parathyroid administration is not related to the diuresis which eventually occurs.

Parathyroid hormone increases the $[Ca^{++}]$ of the blood plasma (Mc-

Lean, Barnes and Hastings, 1935). Gilligan, Volk and Altschule (1933) found that the calcium increase of edema and ascitic fluid (which contained very little protein) was similar to that of the blood plasma. Cantarow, Brundage and Housel (1937) found proportional increases in the diffusible and nondiffusible calcium of the blood plasma. Cantarow, Haury and Whitbeck (1938) consider that the parathyroid hormone may increase the rate of diffusion of calcium from the blood to the tissue fluids.

Parathyroidectomized rats show an increased desire to ingest calcium, as is indicated by the results of Richter and Eckert (1937). When offered a choice of water or calcium lactate solutions, the operated animals enhanced their calcium supply by a greater intake of the calcium lactate.

Cantarow, Brundage and Housel (1937) found that parathyroid hormone decreases the phosphatase of the blood stream, which is opposite from the effect of large doses of vitamin D. Hansen, MacQuarrie and Ziegler (1938) reported that parathyroid hormone, or large doses of vitamin D, depressed the blood phosphatase of patients suffering from osteogenesis imperfecta, and caused a negative calcium balance similar to that produced in normal persons.

Relation of calcium supply to parathyroid secretion. Under certain conditions, the level of the blood calcium may not be a criterion of the state of activity of the parathyroid gland. When the calcium balance is negative and the calcium store in the bones is depleted, such as occurs in rickets, more than the normal amount of the parathyroid hormone may be required to keep the blood calcium normal. Hyperplasia of the gland as a whole and hypertrophy of the cells were first noted in rachitic rats by Erdheim (1914). Since that time, increase of the size of the gland in human rickets has been noted by Ritter (1920) and by Pappenheimer and Minor (1921), who found the increase due to multiplication of the cells. Administration of large amounts of vitamin D to rachitic patients (Albright and Sulkowitch, 1938) increased the plasma inorganic phosphate, suggesting that the activity of the parathyroids had been decreased. This increase of plasma phosphate was not observed in patients suffering from idiopathic hypoparathyroidism.

Hyperplasia of the parathyroids was produced by feeding a low calcium diet to rats by Luce (1923), and to rabbits by Baumann and Sprinson (1939). In the latter experiments, the blood serum calcium and inorganic phosphate were low, but the parathyroids more than doubled in size and showed hypertrophy of the cells and nuclei.

That enlargement of the parathyroid glands occurs in chronic renal disease has been noted several times—Bergstand (1920), Pappenheimer and Wilens (1925), and Gilmour and Martin (1937). Additional evidence that the parathyroids are connected functionally with the kidneys has been given by Donahue, Spingarn, and Pappenheimer (1937). They found that partial nephrectomy in rats leads to an enlargement of the parathyroid gland, and an increase in the calcium content of the remaining renal tissue. The increase of kidney calcium does not occur if the parathyroids are removed. The suggestion that phosphate retention (with consequent tendency toward low blood calcium) is the common cause of these changes has been made on the basis of observations on patients by Albright, Baird, Cope and Bloomberg (1934). However, this has not yet been proved experimentally on animals. It may be significant that the diet of the rats of Donahue (cited above) had a fairly high phosphate content.

Possible differences between parathyroid extract administration and hyperparathyroidism. The anatomical and histological changes following repeated administration of parathyroid hormone to normal rats does not reproduce exactly the condition of hyperparathyroidism, as is shown by the work of R. B. Burrows (1938). The extracts seemed to give rise to a refractoriness toward the hormone, resulting in an increased requirement in the amount of hormone necessary to prevent a return of the blood calcium of the animals to normal. A condition similar to "marble bone disease," or hypercalcification of the bone was caused by the extract. This was in contrast to the decalcification observed in hyperparathyroidism, and was thought to be the result of an antibody or antihormone elaborated by the animal.

Effect of the thyroid gland. Hyperthyroidism causes a profound increase in the urinary calcium excretion. Aub, Bauer, Heath and Ropes (1928) carried out balance experiments on patients with exophthalmic goiter before and after subtotal thyroidectomy. The patients received about 0.1 gram of calcium in the food per day and excreted 3 to 6 times this amount in the urine alone before treatment was instituted. On the average, this was about 4 times the urinary excretion of calcium by normal persons on the same diet, and 6 or more times that of patients with myxedema. The fecal calcium excretion of the hyperthyroid patients was also greater than that of the normal or of the myxedematous patients.

It may be that the increased rate of metabolism interferes with the absorption of calcium. The diet contained about 8 times as much

phosphate as calcium; and the calcium which entered the intestinal tract by way of the intestinal juices might, therefore, have been precipitated in the intestinal excreta as phosphate. The increased urinary excretion appeared to be a measure of the increased resorption of bone salts. That it is not due to a secondary effect on the parathyroids appears to be supported by the fact that the blood calcium remained normal. Puppel and Curtis (1936) compared the calcium and iodine balances of two women suffering from hyperthyroidism with those of two normal women and a hypothyroid patient. In general, the iodine balance varied directly with the calcium balance. Compared with the controls, the patients with exophthalmic goiter showed a large negative balance of calcium, which, in one case, was accounted for by increased excretion in the urine, and, in the other, by increased fecal excretion. Hansman and Fraser (1938) found that irradiation of the thyroid region decreased the negative calcium balance of hyperthyroidism. They concluded that hyperplasia of the parathyroid may be associated with hyperthyroidism and is responsible for the negative calcium balance.

Severe hyperthyroidism in rats produced by feeding desiccated thyroid does not produce decalcification of adult rats or any evidence of lack of calcification of rapidly growing rats (Smith and McLean, 1938). The wet weight of the femurs of the thyroid-fed rats was greater than that of the controls, but the increase was due to a higher water content. The thyroid feeding may lead to premature cessation of growth as a consequence of endochondral bone formation. Coryn (1937) described changes in the cartilage of rabbits resulting from thyroidectomy and the administration of thyroid.

H. Hanke (1936) found that total removal of the thyroid of guinea pigs had no effect on the healing of bone fractures. Todd, Wharton and Todd (1938), from a comparison of the skeletons of twin sheep, one of each of which was thyroidectomized, concluded that the changes observed resulted from a diminished rate of growth with no prolongation of the growth period. There was no modification of bone texture, weight, or thickness.

Effect of pregnancy and sex hormones. The influence of the sex hormones on the blood calcium has been actively studied by many investigators. The results often seem to be contradictory, as noted by Marlow and Koch (1938) in reviewing the literature on this subject. Much of the difficulty results from the fact that the total blood calcium concentration represents the net result of 3 processes which affect it. These are: 1, the metabolic balance between the supply of and demand

for calcium; 2, the regulation of the calcium ion concentration of the blood plasma by the parathyroid, and 3, the strictly chemical equilibrium between calcium ions and protein ions in the blood plasma. Until the effect of the various sex hormones on each of the above processes is known, their effect on the blood calcium will not be clearly understood. We shall, therefore, summarize only briefly some of the more striking changes which have been noted, but their interpretation must await further work.

Pregnancy. Oberst and Plass (1932) and others noted that the blood calcium and inorganic phosphate were usually low in pregnant women. Finola, Trump and Grimson (1937) gave $CaHPO_4$ and viosterol to 33 women and found that the blood calcium increased during the latter stages of pregnancy. Twenty-five others, who received no treatment, showed a decrease in the blood calcium. The infants born to the treated group showed more dense calcification.

Ramsay, Thierens and Magee (1938) determined the calcium and phosphate of 95 women during the seventh month of pregnancy and found the calcium to be abnormally low (below 9 mgm. per 100 cc.) in 39 per cent of the cases.

M. Bodansky (1939) and M. Bodansky and Duff (1939) observed the variations of the blood calcium during the course of pregnancies of 300 women. A slight decrease of calcium and inorganic phosphate usually occurs as pregnancy advances. This decrease is not referable to changes in protein or water content, and is considered to represent the balance between the demands of the fetus and the supply of the mother. It is also considered that hyperfunction of the parathyroids exists in spite of the lowered blood calcium in pregnancy, and that hyperfunction is necessary to maintain the blood calcium above the tetany level. Pyle, Potgieter and Comstock (1938) found a correlation between the blood calcium level and the net calcium balance as well as between the blood calcium level and the calcium excretion during pregnancy.

It was found by Y. Sato (1938) that calcium injected into the pregnant rabbit passes into the placenta, thence into the fetus, but calcium injected into the fetus will not return to the blood of the mother. Parathyroidectomy did not lower the blood calcium of the fetus. The blood calcium during the first 4 days of life is 10 per cent lower than that of the cord blood and tends to rise to the prenatal level in the next 5 days (Denzer, Reiner and Vogel, 1938). H. Bakwin (1937) considers that tetany of the newborn results from hypoactivity of the parathyroids.

Estrins. A slight temporary increase of blood calcium occurs in dogs

about a month after each period of estrus (Cheymol and Quinquaud, 1937). These investigators found (1938) only a transient increase in blood calcium after administration of estradiol benzoate to 3 female dogs and testosterone propionate to 3 male dogs. The blood calcium of rabbits increases about 25 per cent after ovariectomy and returns to normal on the administration of estrone (T. P. Storbecker, 1937).

Levin and Smith (1938) found very little effect after administration of large doses of theelin or dihydrotheelin, or after ovariectomy in rats and rabbits. Theelin increased the serum calcium in rats, doves, fowl, and dogs, according to Riddle and Dotti (1936).

Marlow and Koch (1938) investigated the effects of an estrogenic preparation from pregnancy urine and hog ovaries on the blood calcium level of fowls, rats and rabbits. No change in blood calcium was found except for a slight lowering in gonadectomized rats, and a tendency to rise after administration of ovarian estrogenic preparations. Huey and Marlow (1938) tested each fraction obtained during the preparation of the purified ovarian extracts, but found none with blood calcium raising properties. Estrin injected into infantile guinea pigs resulted in premature calcification of the long bones, and consequently, stunting of the animals, according to the results of H. Seeman (1937).

With the exception of the bones in the immediate vicinity of the pubic symphysis, the marrow of the skeleton of rats was almost completely filled with endosteal bone after they had been given 250 international units or more of estrins (hydroxy estrin benzoate, equilin benzoate) per week for 8 weeks (Gardner and Pfeiffer, 1938). These investigators found also that administration of 1000 units of estrogen daily to pigeons increased the blood serum calcium more than 100 per cent. The estrogen caused hypercalcification of the long bones of the males, similar to that which occurs normally in the females at the time of ovulation (Pfeiffer and Gardner, 1938). B. C. Mocola (1937) found small decreases in the inorganic and total phosphate of the femurs of rats after giving 12 to 40 units of estrone daily for 2 to 3 months.

Pituitary. Removal of the hypophysis alone had little influence on the blood calcium of dogs. However, dogs deprived of both the parathyroid and hypophysis were more susceptible to tetany than those lacking only the parathyroids, according to Speranskaya-Stepanova (1937). Hypophysectomy had no effect on the serum calcium of rats (Anderson and Oastler, 1938, 1939). On low calcium diets, the hypophysectomized rats showed a lower blood inorganic phosphate than did the controls.

Alkaline extracts of the anterior pituitary may have parathyrotropic

activity. Friedgood and McLean (1937) found an increase (about 10 per cent) of the blood calcium when such an extract was administered to guinea pigs for 9 days; but the blood inorganic phosphate was not changed by this procedure. Riddle and Dotti (1936) found that prolonged (5 to 7 days) administration of the gonad-stimulating hormone increased the serum calcium 30 per cent or more in normal, hypophysectomized or thyroidectomized pigeons, but not in castrates.

The calcium deposition produced in the kidneys of dogs by parathyroid extract can be enhanced by administering it together with the pituitary growth hormone. The renal calcification is also favored by a carbohydrate diet (R. C. Moehlig, 1938).

Silberberg and Silberberg (1937) found that ovariectomy of growing guinea pigs caused hyperplasia and hypertrophy of the epiphyseal cartilages which could be modified by administration of an acid extract of the anterior pituitary.

Follicular hormone. Treatment of normal rats for 44 days with follicular hormone caused a twofold increase in the blood calcium and a decrease in the calcium content of other tissues, particularly the skin and muscle. This change was accompanied by a large negative calcium balance (Bach, 1937). Luigi DiBella (1937) found that prolan increased the calcium excretion of frogs.

Miscellaneous effects of other glands. Donati (1938) found that unilateral adrenalectomy in rabbits caused a drop in blood calcium in 4 to 10 days. Sandberg, Perla and Holly (1937) found no change in the calcium or magnesium excretion following suprarenalectomy in rats. Lucke and Wolf (1938) claim that either cortical suprarenal extract or vitamin C, when injected into dogs, produced, after several days, a positive calcium balance.

Removal of the thymus from chickens (G. Maughan, 1938) or dogs (Ornstein and Lucenescu, 1938) has no effect on blood calcium or calcium metabolism. S. Tsujioka (1937) finds that feeding a spleen extract decreases the blood calcium of thyroparathyroidectomized and splenectomized dogs. A. Beretta (1938) finds that the blood calcium is increased during insulin hypoglycemia. Sato (1938) reports that, as the mammary glands of the rabbit enlarge, a substance appears in the blood stream which has the power to lower the serum calcium of the normal rabbit. He also finds that an extract of the parathyroid of the cow fetus contains a calcium-raising principle, and the thymus and spleen contain a calcium-lowering principle.

CONCLUSION

The foregoing discussion of recent contributions to our knowledge of the physiological and chemical factors which are concerned with calcification is, unfortunately, often rather fragmentary. The relation between the calcium and phosphate ion concentrations of the blood plasma and the deposition and solution of calcium phosphate salts in bone has been discussed in detail because of the recent modifications which have occurred in our knowledge of this relationship.

One may summarize the present evidence by stating that the calcium and phosphate ion product of blood plasma is normally as high as it is possible for it to be without initiating spontaneous precipitation of calcium phosphate salts.

The factors which make it possible for this ion product to be maintained at a maximum value are physiological—i.e., the balance existing between the processes of absorption and excretion. At present, our knowledge of how the various hormones and vitamins affect these physiological processes is very imperfect. It is hoped, however, by pointing out in the present review the nature of the problem still confronting us, and the character of the observations which must be taken into account in any adequate description of calcification, that further investigation on the subject will, thereby, be aided.

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FAT ABSORPTION AND ITS RELATIONSHIP TO FAT METABOLISM

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The composition of the diet and the mechanism of absorption may determine the ultimate destination and fate in the animal body of absorbed fat. Recent work makes it possible to indicate some of the interrelations, and throws doubt upon the validity of certain hypotheses which are generally accepted at the present time. As the nomenclature of fats is often confusing, the basis for the terms used in this paper is shown in table 1.

In table 1 the more general terms used in this paper are shown at the top and their progressive particularisation is indicated.

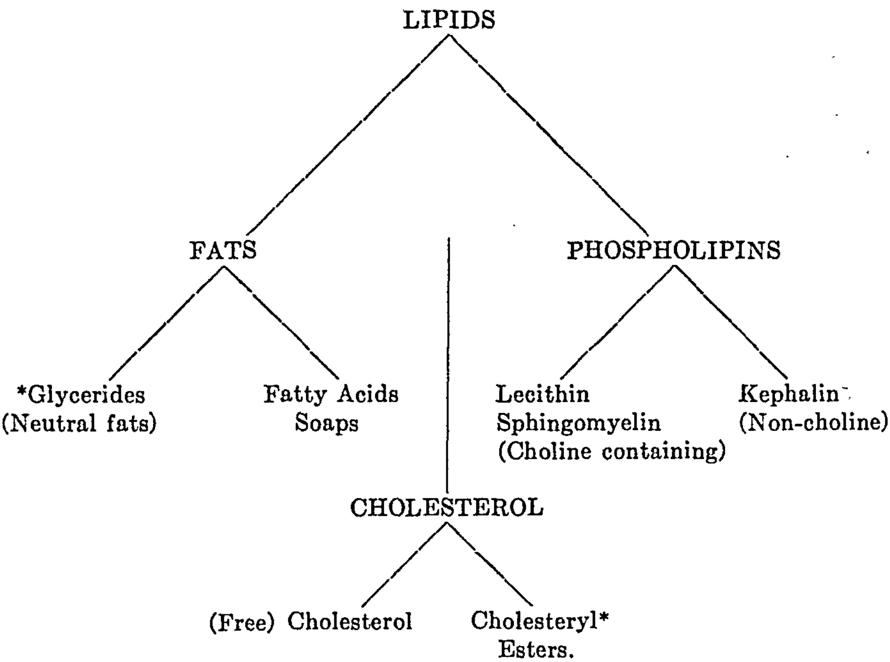
During the last seventy years observations have been recorded by workers in all parts of the world using a diversity of animals under a variety of experimental conditions. Many experiments have been made using herbivores, whose fat intake is normally low, many others have been conducted on carnivores with fundamentally different living conditions, diet and needs, and but few on the human subject. From this heterogenous mass of material the general conception of fat absorption and metabolism in the animal body has evolved. There have been many special difficulties to overcome in the elucidation of problems of fat absorption and metabolism. Rapid changes in the fat content of the blood are not easily demonstrable by methods involving chemical analysis as relatively large amounts of blood are required for this purpose. The literature contains many anomalous statements which originate from conclusions based upon the analysis of infrequently collected blood samples. Recently, the use of chylomicrographs, which show the number of fat particles seen in the blood with a standardised dark-ground microscopic technique, has made serial investigation of the blood fat easier. The changes demonstrated by this method are sufficiently great to be significant but the quantitative relationships can only be regarded as relative at present.

Another difficulty has been to determine the origin and destination of

fat detected in the blood or that accumulating in the tissues. The labelling of fat with deuterium or elaidic acid, and of phospholipin with an identifiable isotope of phosphorus has made it possible to trace these lipids from one part of the body to another.

Recent work on the development of fatty livers opens up new possibilities. The effects of choline, protein and other lipotropes upon the fat in the liver are very complex and many points require clarification

TABLE 1



* Glycerides = triglycerides unless otherwise stated.
Cholesteryl esters = esters of cholesterol and fatty acids.

before the full significance of these observations can be appreciated. To exclude variable factors or provide sufficient materials for analysis, it has often been necessary to devise experimental conditions that are far removed from normality. The feeding of gross amounts of abnormal fats, the interference with normal intestinal function and equilibria, the upset of intra-intestinal pressures and the administration of cellular poisons in excessive doses are but a few of the procedures which may have led to confusion in the interpretation of results. Although

a wide basis of experimentation is highly desirable and various experimental procedures may be necessary in order to emphasize and demonstrate one particular factor in the mechanism, the results of such work demand most careful consideration if a correct assessment of the mechanism in the normal intact animal is to be reached.

DIETARY AND INTESTINAL FACTORS IN FAT ABSORPTION. *Dietary factors.* Deficiency of vitamins A, B or D causes a decrease in the rate of absorption of fat (1) probably due to general impairment of nutrition rather than any specific effect upon fat absorption. In certain animals a more direct effect of the vitamins upon fat absorption can be demonstrated. Extensive esterification of fatty acids with cholesterol and vitamin A is found in the small intestine of the halibut (2). On a vitamin B deficient diet rats show a remarkable craving for fatty food (3) and histologically there is an alteration in the distribution of fat in the intestinal cell (4), an effect also seen in vitamin A deficiency. The normal fine dispersion of the fat globules with grading in size from without inwards is replaced by irregular grouping of large masses of fat. The significance of these histological changes has never been determined. Other effects of vitamins appear to be more on the metabolism than the absorption of fat.

A surplus of neutral fat in the food results in delayed emptying of the stomach, and great excesses may cause vomiting, so that under normal circumstances it is impossible for more than carefully regulated quantities of fat to pass into the small intestine at one time. Glycerides in the food are mainly mixed triglycerides which have a lower melting point and are much more easily absorbed than the simple triglycerides of the higher saturated fatty acids containing only one type of fatty acid. The melting point is lower after recent melting, thus cooking may make certain fats more assimilable.

Cholesterol in the food will not be absorbed unless fat is also present (5) in a quantitative relationship. In fishes (2) the esterification of cholesterol and fatty acids in the intestine may assist in fat absorption. The effect of lipase and bile salts upon esterification of cholesterol appears to vary under differing experimental conditions (6) (7) (8) but their influence is probably a factor in this mechanism. Increase or deficiency of lecithin in the food does not affect fat absorption to any great extent (9) although effects may be masked by the presence of phospholipin in the bile.

Intestinal factors. It has been stated (10) (11) that fat must be hydrolysed before it can be absorbed, and this premise has been used

in the interpretation of many experiments. For several reasons, however, this lipolytic hypothesis must be regarded as unproven.

Lipase is found in the pancreatic juice of most animals and active lipolysis can be demonstrated in the upper part of the small intestine. The presence of lipase in other intestinal juices is more doubtful. Gastric lipase does not exist in significant amounts in the adult stomach, and, in any case, it can play no part in the preparation of fat for absorption. There is no evidence that fat passing through the stomach undergoes any lipolytic change, and normal gastric acidity precludes lipolysis. Lipase is absent from the succus entericus of the cat (12). The contrary evidence (13) based upon the occurrence of lipase in mucosal extracts cannot be accepted since this is not evidence that active lipolysis occurs in the lumen of the intestine, the absence of which can be demonstrated. Intestinal contents removed from a fistula anywhere in the lower part of the cat's small intestine show no signs of lipolysis, but active fat absorption can be demonstrated below the site of the fistula. Lipase passes into the intestine with the early reflex flow of pancreatic juice. Its hydrolytic action soon comes to a stop even when the fat is very finely dispersed (14). Lipase forms adsorption complexes with calcium and bile salts which activate it in an alkaline medium but inhibit it if the medium is acid (15), which is the normal reaction of the intestine except for the upper part of the duodenum (16). It is, therefore, only in the duodenum that lipase can work under anything but adverse conditions. The addition of extra lipase to a known amount of ingested fat results in a marked decrease of the post-absorptive lipemia (17) proportional to the amount of extra lipase added. The fat is still absorbed but, in some way, the extra lipase prevents its appearance in the systemic circulation. The ingestion of neutral fat produces different results from those obtained with an equivalent amount of fatty acid and glycerol (18). If olive oil and oleic acid, both stained equally with Sudan IV, are given with their food to two groups of rats for a period of 10 days, the animals receiving the glyceride have deeply stained fat depots, whereas those taking the acid show practically no depot staining. Fecal analyses show that the fats are equally absorbed in both groups and the weight curves of the two are identical. Similar results have been obtained with rabbits. In anesthetised rats the lipids can be injected into the intestine and simultaneous systemic and portal blood specimens obtained. If glyceride is introduced, the systemic blood shows the normal increase of fat particles and there is but little change in the portal blood.

If fatty acid and glycerol are injected, the portal specimens show a definite increase in the number of particles but the systemic blood does not. In agreement with this, the lacteals only appear milky when neutral fat has been placed in the intestine. Glycerides and fatty acids have been given to human subjects by duodenal tube and similar systemic phenomena observed. It is difficult to reconcile these findings with the supposition that all neutral fat must be hydrolysed into fatty acid before it can be absorbed. Perhaps unhydrolysed fat passes by the thoracic duct and systemic circulation direct to the fat depots while several alternative possibilities await the fatty acid fraction.

Stained preparations of intestinal cells show definite changes during fat absorption (19). After 30 minutes the cells contain droplets which stain with Sudan and also give a positive reaction with the Fischler technique. After 6 hours the cell is found to be full of Sudan-staining material which is Fischler-negative. This suggests that in the first specimen the cell contains fatty acid but in the second neutral fat, although considerable doubt has been thrown upon the validity of the Fischler method of differentiation between fatty acid and glyceride (20). This has been interpreted as evidence in favour of resynthesis of neutral fat from fatty acid and the lipolytic hypothesis. There is no reason why the neutral fat in the second specimen should have been derived from fatty acid demonstrated in the first specimen; it is quite possible for the neutral fat to have been absorbed in the unhydrolysed form to produce an identical histological picture, and, under no circumstances, can this evidence be regarded as demonstrating the essential nature of lipolysis.

Although a rough parallelism exists between the action of lipase on triglycerides of higher fatty acids and their rates of absorption, there are several other factors such as melting point and solubility which can equally well account for the differences. The non-absorption of paraffins is not necessarily due to the impossibility of hydrolysing them with lipase. There are numerous examples of differential absorption of substances much more closely related chemically than paraffins and glycerides.

It may be concluded that lipolysis does occur, but it is unjustifiable in the present state of our knowledge to state that this is an essential preliminary to absorption of glycerides. It may be that the fact that lipolysis is only partial will prove an important determining factor in fat metabolism.

There is no doubt that taurocholic and glycocholic acids or their salts

are important for the absorption of fat. It is not always possible to demonstrate that bile salts facilitate the passage of fatty acids through artificial membranes (21) (22) but there is considerable evidence that bile salts and fatty acids form water-soluble complexes and that this hydrotropic action is of fundamental importance in determining the passage of fatty acid through the intestinal wall.

A surprising shortage of bile salts is evident, if all the fat absorbed from the intestine passes in the form of water soluble complexes of fatty acid and bile salt. Making every allowance in their favour, the bile salts cannot carry through more than 30 grams of fat per diem in the form of fatty acid, a quantity far short of the amount known to be absorbed. Six hours after injection of bile salts into an isolated loop of intestine rather less than 10 per cent of the amount injected can be recovered from mucosal scrapings. This is interpreted (23) as evidence that the bile salts are adsorbed to the surface of the intestinal mucosa, from which vantage point they are alleged to carry on their hydrotropic functions without being circulated. A simpler explanation might be that all the fat is not absorbed due to the hydrotropic action of the bile salts—fatty acid enters thus but the remainder passes in as neutral fat, a process which does not involve a quantitative relationship with bile salts.

In addition to their hydrotropic properties the bile acids cause a marked lowering of surface tension which will favour the formation of a finely dispersed oil-in-water emulsion. The presence of a stabiliser is essential if emulsification is to be effective, as otherwise a rapid coalescence of the globules will occur. A stabilising film on the surface of the oil globules could be furnished by either protein or soap. Small amounts of the latter can still be present even if the contents of the intestines are slightly acid. The fine dispersion of fat, while allowing more rapid initial hydrolysis, may also be a part of the mechanism for glyceride absorption.

The effect of phospholipin and cholesterol upon fat absorption was considered with their possible inclusion in the diet. The presence of these lipids in the bile should not be forgotten.

Fat can be absorbed from the small intestine of the cat (12) without any sign of active lipolysis or upset of normal intestinal function or intraintestinal equilibria. Neutral fat passes from the intestinal cell to the lacteal and it permeates many cell membranes in the body without any question of preliminary hydrolysis. In all these cases it must be in a state of fine dispersion, possibly molecular division. It may

pass through the intestinal cell from the lumen of the gut in a similar manner. The bile salts do not exert any hydrotropic action upon neutral fat, but in the presence of stabilisers bile salts will promote emulsification. It seems improbable that dispersion would be fine enough to allow passage through a cell membrane, and histological preparations suggest that the fat enters in a state of molecular division and forms larger aggregates and globules deeper in the cell. After emulsification, the bile salts may act as a "wetting agent" allowing finely dispersed oil droplets to come into intimate contact with the lipid membrane of the cell. The neutral fat molecule is large but certain protein molecules can pass unchanged through the intestinal wall, giving rise to allergic phenomena. The lipid nature of the cell membrane will facilitate the passage of the fat molecule. The rapid permeation of finely dispersed oils into animal tissues in the presence of a suitable "wetting agent" can be readily demonstrated and is made use of industrially. The exact nature of the absorptive mechanism requires further investigation.

THE FATE OF ABSORBED PRODUCTS IN THE INTESTINAL CELL. The fat in the thoracic duct lymph mainly consists of neutral fat, and therefore, if the lipolytic hypothesis is correct, resynthesis of glycerides from absorbed fatty acid and glycerol must occur in the intestinal cells. If, however, all the fat is not hydrolysed before absorption, resynthesis ceases to be an essential part of the mechanism.

The recovery of triglycerides from the thoracic duct after feeding with monoglycerides or fatty acids, and other classical experiments suggest that, under special conditions, resynthesis of glyceride may occur in the intestinal cell. The derivation of these triglycerides from other sources than the ingested material has never been properly excluded and the experiments were not carried out on a quantitative basis. They show perhaps that a mechanism exists whereby resynthesis can occur, but they do not demonstrate that it is either normal or essential. The chemistry of the resynthesis remains obscure. Given suitable conditions for the reverse reaction to occur, lipase might be expected to fulfil a catalytic rôle, but not much attention has been paid to this possibility. An intermediate stage of phosphorylation has been suggested (24). Addition of glycerophosphate to fatty acid in the intestine is said to increase the amount absorbed in 6 hours. With careful assessment in rats under normal conditions no significant effect of glycerophosphate on the rate of absorption of fat could be demonstrated (25) and no effects can be detected in studies of human fat absorption. If rats are injected with 0.07 to 0.1 mgm./gram body weight of moniodo-

acetic acid there is considerable interference with fat absorption. Somewhat similar results follow injection of phlorrhizin in doses of 50 to 100 mgm. per 200 grams rat. These doses are large and the results might be accounted for by other explanations than interference with phosphorylation processes. This dose of moniodoacetic acid damages the intestinal cells and interferes with the absorption of sodium and xylose (26) and the effects of phlorrhizin can also be shown to be due to cellular damage and not to any specific effect upon phosphorylation (27).

Slow absorption of fatty acid occurs in some of these experiments, but no neutral fat accumulates in the cells. This was thought to be due to interference with resynthesis, but in view of the severe cellular damage it is possible that the water-soluble complexes gradually permeate the intestinal wall but the neutral fat cannot pass through since its absorption depends upon the normal lipid structure of the cell.

Adrenalectomy causes slowing of the absorption rate of fat, normality being re-established by injection of a potent cortical extract (28). The effect has been attributed to control of phosphorylation processes by the cortical hormone similar to that described by the same workers in connection with carbohydrate absorption (29). The absorption of carbohydrate is normal, however, if the health of the adrenalectomised animals is maintained by sodium chloride therapy (30). On the available evidence it is not possible to link phosphorylation with triglyceride resynthesis.

There is, however, evidence that fatty acids may undergo phosphorylation, not as an intermediary in glyceride resynthesis, but in the production of phospholipin. Experiments with unsaturated fats (31) suggested that the intestinal cells were able to form phospholipin from absorbed fatty acids. Later this was confirmed by using iodised fats (32) and elaidic acid (33) to trace the fatty acid from the ingested material to the formed lecithins. Further support is afforded by experiments tracing the phosphorus fraction by the use of labelled phosphorus (34).

Unsaturated fatty acids are preferentially selected for phospholipin synthesis, but with adequate supplies of fatty acid relatively saturated and unsaturated fatty acids are selected alternately (35). Elaidic acid may replace as much as 30 per cent of the natural fat in the formed lecithins. Elaidin turnover in the liver is very rapid but in the tissues it is slow (36). This may be due to the more unsaturated phospholipins being used for cell construction in the liver and tissues whereas the more saturated phospholipin is for metabolic use in the liver (37).

According to Leathes' hypothesis phospholipin is an important in-

termediary in fat metabolism. This mainly concerns lecithin formation in the liver, while these recent investigations show its formation in the intestinal cells (38). The presence of fat in the diet is essential for formation of phospholipins in the intestine but is without effect upon their synthesis in the liver (39). In the liver, formation of lecithin may be either from fatty acid or glycerides but in the intestine it may be formed from fatty acids only. These two sites of phospholipin synthesis are quite independent of each other and may fulfil quite different functions for the body as a whole. The formation in the liver may be regarded as being mainly endogenous and concerned with metabolic utilisation of fat whereas the intestinal mechanism is exogenous and is involved with fat transport; probably both play a part in cell construction.

The close association between cholesterol and fat in the diet and their absorption has already been discussed. Whether cholesteryl esters are formed in the intestinal cell or in the liver is not known. The effect of the bile and lipase upon cholesterol esterification is the subject of contradictory reports, but there seems to be no essential reason why these cholesteryl esters should not be formed in the intestinal cells, and they even form in the intestine in some animals. If this is the case some of the absorbed fatty acid will be taken up in this way, and the formed cholesteryl esters may pass to the liver in the portal blood.

Some fatty acids may pass direct to the liver as water-soluble complexes with bile acids, and a small amount may form soaps, which can be detected in the chyle, where they may form surface films on the fat globules.

It seems possible that the greater amount of neutral fat in the intestinal cell may have been absorbed unhydrolysed in a molecularly dispersed form. This fat passes out of the cell to the lacteal and thence into the systemic blood to the fat depots. It is possible that some modification of its contained fatty acids may occur, such as alteration in saturation or length of the fatty acid chains (40).

Transport of fat in the blood. The transport of fat in the animal body has been recently reviewed (11), consequently it is only necessary here to deal with new points or those having a special bearing on this discussion. Fat absorbed from the intestine may be transported in the form of neutral fat, phospholipin, cholesteryl esters, soap or free fatty acid. It can pass by the portal vein direct to the liver or it can travel by the intestinal lymphatics and thoracic duct into the systemic circulation, short-circuiting the liver.

Neutral fat occurs in the blood as a finely dispersed emulsion with fat globules ranging in diameter from 35 mμ. to 1 μ. When studied under dark-ground illumination the blood is seen to be full of brightly illuminated particles. Some evidence suggests that they have a protein structure (41) but with fuller investigation their biochemical and physiological properties show that the main bulk of the particle is neutral fat. This is undoubtedly true of the bright particles, and on the outside of this particle is an adsorbed layer of globulin which gives it properties with precipitants and electrophoresis (42) which may have led to the erroneous idea that the whole particle was formed from protein. This protein may be secondarily adsorbed on to a soap film which it will protect against disruptive effects of the salts of the plasma. Artificial particles can be prepared with this structure and they behave in every way identically with chylomicrons (43).

By the use of carefully standardised technique it has been possible to estimate the number of particles in a standard field following the ingestion of a known amount of fat. These investigations can be made on a drop of blood and consequently specimens can be collected as frequently as required. A chylomicrograph is constructed from the counts which shows the relative changes occurring in the blood fat during the course of the experiment (43).

The normal chylomicrograph after a meal containing fat is a composite curve consisting of an initial and a delayed rise. The former starts within 15 minutes of ingestion of the fat and lasts up to 1 hour whereas the latter does not start until this period has elapsed. The initial rise is due to passage of fatty residua of the previous meal into the blood stream and shows the great importance of careful preparation of the animal prior to experiments on fat absorption. The delayed rise is due to actual absorption and after a normal mixed meal in the human subject it reaches a maximum in 2½ hours and the basic level of blood fat is regained in 4½ hours (44). The figure of 6 hours as the time for maximum lipemia is false. It was presumably based upon observations made after meals containing an excess of fat which cause a marked delay in the stomach emptying time.

It has been shown that phospholipin in the form of lecithin is an important method of fat transport. The sphingomyelin and kephalin fractions do not appear to be involved. The lecithin is not visible under ordinary dark-ground illumination and it does not appear to enter into the structure of the chylomicron. Bound cholesterol in the form of cholesteryl esters of fatty acids may play their part in fat trans-

port. The formation of these unstable compounds is influenced by lipase and bile salts. Only small amounts are detected in the chyle but there is a significant increase both in the liver and the systemic circulation during fat absorption.

Distribution of lipids between systemic and portal circulations. It has often been pointed out that water-soluble absorption products pass into the portal blood to the liver while those that are not in aqueous solution travel in the lymphatics to the thoracic duct. Various hypotheses have been put forward (45) to explain the divorcing of the fatty acid/bile acid complex in the intestinal cell. There is, however, no convincing evidence to show that either acidity or the presence of positively charged ions in the intestinal cells brings about this change. Some, at least, of this water-soluble complex might be expected to pass up to the liver with other substances in aqueous solution. It has been suggested that lower fatty acids tend to escape resynthesis and may pass to the liver in the portal blood (46). The close association of cholesterol and fat in absorption, the increase in the liver of cholesteryl esters, which are essentially exogenous and not due to transport of cholesterol from other tissues (47), and the lack of their detection in the chyle, suggest that in this form also fatty acid may pass directly into the portal circulation. Since Munk failed to recover 40 per cent of absorbed fat in his human experiments from a lymphatic fistula, it has always been a matter of doubt as to whether all the absorbed fat travels by way of the thoracic duct. Analyses of portal blood have brought conflicting reports mainly due to examination of infrequently collected samples. From experiments on rats and human subjects (18) it seems possible that a partition of absorbed fat occurs which may be predetermined by lipolysis. The fatty acid fraction passes to the liver possibly in solution due to the hydrotropic action of the bile salts, or as cholesteryl esters, and the glyceride portion takes the lymphatic pathway to the depots. Possibly not all the fatty acid is destined for the liver since some at least will form phospholipin in the intestinal cells and this may travel by either route.

THE DESTINATION OF ABSORBED FAT. *Fat depots.* The fat depots are collections of adipose tissue laid down in various parts of the body for storage purposes, which can be called upon by the body when required. The main depots are in the subcutaneous tissue, the omentum and round the viscera. The fat in the liver is not included since it is essentially for metabolic use. The fat in the depots is mainly triglyceride of low iodine value and bears some relationship to the ingested fat. Controversy has raged over the question of whether the fat in

the depots is identical with that ingested. The iodine values and chemical constitution of fats have been taken into account in trying to solve this problem. The resulting observations are conflicting which is not surprising since fat may be formed from carbohydrate, be derived from other sources or modified after absorption, so that fat from the food may be slightly altered or diluted by other fats. Little is known of the factors controlling these various changes so that in each experiment different effects may occur which cannot be assessed. There is no doubt that in the normal animal on a mixed diet most of the fat deposited in the depots is derived directly from absorbed fat. In eels fed upon mussels with low fat content the depots remain unaltered whereas if they have herring flesh the depots approximate closely to herring fat in character (48). This relationship between the depots and a high fat content in the diet may be significant, for in rats also a greater constancy in the composition of the depot fat is found with a low fat diet (49). In many grazing animals the consistency of the fat can be altered by methods of feeding. In rats stained fats or fat labelled with deuterium or elaidic acid can be traced to the depots. In human subjects there is a marked difference between the blood arriving at the tissues and that leaving. There can be no doubt that fat comes from the intestine to the depots, frequently scarcely altered in character especially when there is a surplus of fat in the food. If there is not much fat in the food then differences are more likely to occur since the dilution will be greater and modification, resynthesis, or formation from carbohydrate is more likely to take place.

There must, of necessity, be a turnover of fat in the depots. With every fat-containing meal more glyceride is removed from the blood into the depots. If there was not a corresponding removal or usage by the depots, they would continually increase in size. That usage does not occur is suggested by the poor blood supply of the depots and the low iodine value of the contained fat. There must therefore be a wastage into the blood stream which probably remains fairly constant except in starvation when it will be enhanced. Deuterium labelled fat and elaidin have been used as a means of studying this turnover in the depots (50) (51). It can be demonstrated that there is a steady turnover of the fat and the half life-time of the fat in the depots is about 6 to 8 days. Other workers have found a less rapid turnover (52) but obviously the rate of wastage will be affected by various factors such as the original size of the depots, the demands of the individual and numerous endocrine factors.

In human subjects starving for seven days, the blood fat level is

basic after a period of 36 hours. Sudden marked increases in the number of particles occur at more or less regular intervals of about five hours, consisting of a change from 5 to 10 particles per field up to 500 or more. This sudden lipemia does not last for more than one hour and consequently unless specimens are taken at least at hourly intervals it may be completely missed. The source of the particles *must be other than the chyle, and they have been termed "lipomicrons"* to distinguish them from the "chylomicrons" which occur after the absorption of fat. Whether this increase of lipomicrons, or "fat crisis," occurs without starvation is not known, but from the fact that such irregularities have not been detected in several hundred post-absorptive studies and that they do not occur until some time after starvation has begun suggests that they represent the enhanced wastage from the depots consequent upon the increased metabolic demand by the body.

The other factors that influence the mobilisation of fat from the depots do not come within the scope of this paper, but they require mention as some interrelations will eventually be found between these factors and those under discussion. Section of nervous paths either in the brainstem or peripheral system modifies the mobilisation of fat from the depots which may be due to the presence of a central controlling mechanism in the mesencephalon (53). The pituitary gland influences fat mobilisation and potent extracts can be shown to have a definite effect upon the blood fat (54). In cases of pituitary dysfunction obesity is often a striking characteristic. Changes in the fat depots occur in *hypo- and hyperthyroidism, but the mode of action of thyroxine in this* connection is not known. The adrenal cortex seems to play some part in fat absorption and emaciation is one of the cardinal signs of Addison's disease. Sexual differences in fat deposition suggest that the gonads are also concerned in this intricate endocrine control. The effects described can be due to direct action upon the absorption, deposition or utilisation of fats or may be concerned with the conversion of carbohydrate to fat.

Liver. When animals are fed on fat containing deuterium, it is possible to demonstrate that the lipids are rapidly taken up by the liver (55). Although modification of fats and synthesis of phospholipins can be shown to occur in other tissues, it is only in the liver that the lipids are prepared by intermediate phosphorylation processes and desaturation for general metabolic use. The liver may also be the site of the conversion of carbohydrate to fat (56).

There are two main classes of lipid in the liver. The first is an

essential part of the cell structure of the gland, which does not vary under ordinary physiological conditions, and the second is metabolic lipid consisting of glycerides, phospholipins or cholesteryl esters, which may vary considerably. The glycerides are derived from the fat in the food which passes through the depots either because it is in excess of the amount that the depots can remove from the blood or because the fat-removing power of the depots is decreased. Glycerides may also be derived from fat which has been previously stored in the depots or be formed in situ from carbohydrate, fatty acids or other substances. The phospholipins may reach the liver after being synthesised in the intestinal cell or they may be formed in situ from fatty acids or neutral fat. Cholesteryl esters may pass from the intestine having been either absorbed in this form or synthesised in the intestinal cell. They may, on the other hand, be formed in the liver from fatty acid.

Accumulation of fat in the liver will occur when the balance of fat income and expenditure is upset. The main sources of fat income are surplus in the diet, diminished uptake by the depots, increased mobilisation from the depots, or increased formation from other sources. Fat expenditure mainly depends upon fat utilisation or alteration in the permeability of the liver cell membranes to the lipids. If liver function is depressed, certain essential procedures in intermediate fat metabolism may be hindered and fat will accumulate. The absence of any essential constituent or catalyst to these intermediate changes might delay fat expenditure. Finally the fat may arrive in greater quantities than the normal liver can deal with, giving rise to a relative hepatic insufficiency. The factors affecting the permeability of the liver cells to lipids are not known.

Simple excess of fat in the diet can easily be shown to cause an increase of fat deposition in the liver. There is an increase in all the fractions associated with metabolism, especially the glycerides. Little is known of the factors affecting the uptake of fat by the depots so that this possibility cannot be assessed. Increased formation from other sources, probably carbohydrate, occurs with aneurin (thiamine) therapy (57), but fatty livers occur with vitamin B6 deficiency (58).

Depression of liver function by poisons is a well-known cause of fatty livers, the fat being brought from the depots. This amount of fat accumulation may be an expression of the normal wastage from the fat depots, but the effect of decreased liver function upon the organism as a whole must be borne in mind since diminished utilisation of carbohydrate might increase the call made upon the depots.

Lipotropy. An interesting group of experiments have been made during the last eight years which introduce a number of new factors into the problems of liver fat. Certain factors have been found to decrease liver fat under conditions that normally result in fat accumulation—these are termed lipotropes.

Depancreatised dogs with severe fatty livers show marked improvement when fed lecithin, and their sugar excretion, which is some index of hepatic function, increases (59). Lecithin also prevents the development of fatty livers in rats on a high fat diet (60). Investigation of the different constituents of lecithin shows that it is the choline fraction which has this effect and many derivatives of choline and choline-containing substances, such as betaine, have a similar action (61). The effect on the liver is a marked decrease in the glyceride fraction and, provided that there is not any excess of cholesterol in the diet, the cholesteryl esters are also diminished. The phospholipin content appears to vary inversely with the glycerides. In the livers of animals fed on a choline-free diet, there is an accumulation of fat, the composition of which can be directly modified by alterations in the lipids ingested (62). The addition of choline to the diet results in dispersal of the liver fat, but it does not influence the degree of saturation of the fatty acids or phospholipin fraction in the liver, nor does it appear to affect mobilisation from the fat depots. From the close association of the ingested fat with the accumulation of lipids in the liver, its action does not seem to be connected with formation of fat from carbohydrate. From consideration of the general principles which govern the deposition of fat in the liver, the effect of choline deficiency should be due either to diminished utilisation or altered permeability in the liver or to decreased uptake of fat by the depots. That choline may play some part in intermediate lipid metabolism, perhaps being involved in phospholipin synthesis in the liver, is an attractive hypothesis. It is strange, however, that the liver should not correct the interference with its function by making use of the many other available sources of choline. There is no alteration in the ratio of choline to non-choline containing phospholipins in the liver (63), and no effect upon the ketogenesis in fasting mice (64) when choline is ingested. When choline derivatives are used, they cannot be traced in the liver phospholipins (65) (66), and normal doses of choline have little effect upon the fatty liver which occurs in starvation (67). There is, therefore, little evidence for supposing that choline stimulates fat oxidation or that it is concerned with phospholipin formation in the liver as a factor in in-

termediate metabolism. It may still, however, stimulate the formation of phospholipin in the intestinal cells. Nothing is known of the effect of phospholipin upon the removal of fat from the bloodstream into the fat depots or upon the permeability of the liver cells to lipids. An adequate amount of lecithin might facilitate the passage of fat into the depots and at the same time accelerate its dispersal from the liver. Deficiency of phospholipin formation in the intestinal cells might then result in more fat passing through the tissues to the liver where it will accumulate. Such a mechanism might explain the main effects of physiological doses of choline. The effect of choline upon the cholesteryl esters may perhaps be explained by the fact that fatty acids may form either phospholipin or the esters. If there is decreased phospholipin formation then cholesteryl esters might be expected to show some increase provided that there is a surplus of cholesterol in the diet. If, however, the formation of lecithin is favoured by the presence of choline and the cholesterol is kept down to a minimum then the balance will swing in the opposite direction.

The amount of protein in the diet may be a factor in preventing accumulation of fat in the liver when there is a surplus of fat in the food. The action is quite different from that of choline, and the glyceride fraction is mainly affected while the cholesteryl esters do not alter (68). Fatty livers have also been produced by ingestion of cystine, an effect which can be prevented by the addition of casein to the diet (69). This antagonistic effect may be due to the lipotropic action of the contained methionine (70). *Methionine causes a decrease of both the* glyceride and cholesterol fractions and the relative lipotropic properties of different proteins are roughly proportional to their methionine content (71). Further investigations seem desirable before attributing the whole of the lipotropic action of proteins to this one factor. No hypothesis can be formulated at present as to how or where the protein exerts its lipotropic effect, but from studies with deuterium-fat a low protein diet can be shown to cause accumulation of fat in the liver which is not derived from the depots but from carbohydrate (72).

Completely pancreatectomised dogs, maintained on an adequate supply of insulin, develop fatty livers which can be prevented by administration of raw pancreas or certain pancreatic extracts (73). It has been alleged that the pancreas contains a hormone, lipocaic, which controls fatty deposition in the liver (74). The evidence for the existence of this hormone is unsatisfactory. The pancreatic extracts have been shown to contain both choline and protein, but various

authorities differ in their assessment of the extracts. Several state that the whole effect of the extract can be accounted for by the choline content (75) (76) (77), but a careful assessment reveals that there may be more action with the extract than can be accounted for by the choline and protein present (78), the former being responsible for one third of the lipotropic action. No account has been taken in these experiments of the possible action of lipase. This will perhaps affect the liver fat, especially the cholesteryl esters upon which raw pancreatic extract has been found to have a marked effect (79) whereas the autoclaved extract has not (80). Until this active principle has been demonstrated in a pancreatic extract which is free from choline, protein and other extraneous lipotropes and the confusing effect of lipase has been clearly determined, a conservative view of the possible existence of lipocaic seems advisable.

Several other factors in the diet may cause an increase of liver fat but practically nothing is known at present of the cause or significance of these findings. If the food contains an excess of cholesterol, there is a marked increase of both bound and free cholesterol in the liver accompanied by an accumulation of glycerides (81). A possible relationship between vitamin A in the diet and fat deposition in the liver has been suggested (82) and fatty livers associated with vitamin B deficiency have already been referred to (58). Addition of lecithin to the ingested food does not appear to affect fat deposition apart from the action of the contained choline (83). Liver or various liver extracts added to the diet result in an increase in the cholesteryl esters in the liver (84). Further investigation must decide how these observations can be correlated with the mechanism of fat absorption and metabolism.

THE EFFECT OF DIET UPON FAT UTILISATION. Apart from the influence of dietary and intestinal factors upon general fat metabolism which has been outlined and which indirectly affects fat utilisation, a more direct relationship between the diet and the metabolism of certain unsaturated fatty acids can be traced. A "fat deficiency" disease can be produced in rats characterised by interference with growth and the occurrence of acrodynia-like skin lesions (85). Cure can be effected by administration of certain unsaturated fatty acids, such as linoleic and arachidonic acids (86). The disease is closely associated with deficiency of vitamins B1 and B6. If aneurin (thiamine) is administered to vitamin deficient pigeons the amount of fat deposition exceeds the supply of ingested fat suggesting that formation of fat from carbohydrate must occur under these conditions (57). Vitamin

B6 is essential for the proper utilisation of the essential unsaturated fatty acids, which cannot be synthesised in the body (87). Deficiency of the essential fatty acids has also been demonstrated in certain cases of eczema in babies, but an adult subject living on a fat-free diet for six months showed no pathological lesions although he was shown to be deficient in essential acids (88). The R. Q. of this subject rose to 1.14 indicating the conversion of carbohydrate to fat, but clearly the essential fatty acids could not be formed in this way. Rats on an equivalent diet developed the typical symptoms of "fat deficiency."

SUMMARY OF THE INFLUENCE OF DIET AND ABSORPTION UPON METABOLISM

Neutral fat enters the small intestine with the food and it leaves it in the same form in the lymphatics, but whether it is broken down and resynthesised as it passes through the intestinal wall has not yet been proved. The lipolytic hypothesis may eventually prove to be correct, but on the evidence available it would seem wiser to bear alternative possibilities in mind, and the dangers of interpreting all other experiments in the terms of a possibly false premise cannot be over-emphasised. One possible alternative is the partition hypothesis, according to which neutral fat is absorbed unsplit and passes by the lymphatic system and thoracic duct into the systemic blood and thence to the fat depots, whereas the fatty acid forms phospholipin, cholesteryl esters or soaps and passes mainly by the portal vein direct to the liver. If this is true, the degree of lipolysis determines how much fat will go to the liver or to the depots. The amount going to the depots will depend upon the relationship of the quantity of fat in the diet to the lipolytic potentialities of the individual, and obesity may be an expression of relative pancreatic insufficiency. The amount of cholesterol ingested affects the formation of cholesteryl esters which is also influenced by the bile salts and lipase. The significance of the cholesterol circulation or the metabolism of cholesteryl esters remains obscure. The choline content of the diet may exert its action upon phospholipin synthesis in the intestinal cells, which in turn may be a factor in removal of fat from the bloodstream into the depots. The mode of action and significance of the other lipotropic factors must await further investigation, but their existence clearly indicates the close relationship between the dietary constituents, intestinal function and fat metabolism.

Not only the quantity but also the quality of the ingested fat affects metabolism. The essential nature of certain unsaturated fatty acids

and the importance of vitamin B6 in their utilisation form another link between the composition of the diet and the ultimate fate of absorbed fat.

The formation of cholesteryl esters or phospholipins, the passage of fat to the liver or its removal to the fat depots, the maintenance of balance between fat income and expenditure, the nutritional health of the body and utilisation of unsaturated fatty acids, all may depend to a great extent upon factors in the diet or the intestine. These are not, of course, the only factors involved since nervous and endocrine action play their part, but it must be left for future work to unfold the possible interrelations between the internal and external controlling mechanism.

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FLUORIDE INTOXICATION

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Recent biological interest in fluorine compounds is due essentially to the following observations: 1, the consumption of a drinking water containing one part per million or more of fluorine during the calcification of the teeth causes a hypoplasia of the teeth (mottled enamel) in man and lower animals; 2, the feeding of certain phosphatic mineral supplements containing considerable quantities of fluorine compounds to domestic animals has affected them adversely; 3, small quantities of fluorides retard fundamental enzymatic processes; 4, in the United States, the use of fluorine compounds (cryolite and barium fluosilicate) as insecticidal sprays for fruits and vegetables has been expanded. General reviews on this subject have been written by DeEds (73), McClure (204) and Roholm (274) (275) (277). The most complete treatise is the monograph by Roholm (273).

This review is concerned primarily with the literature from 1933 to the latter part of 1939. Numerous publications on this subject have appeared during that period and the scope of this review does not permit an evaluation of each paper, hence only representative papers on each phase of the subject will be discussed.

Methods of analysis. Fluoride analyses are still fraught with uncertainties. Some of the data prior to 1933 were obtained by less adequate methods. The analytical data and physiological studies based upon these methods are therefore difficult to evaluate.

The revival of biological interest in fluorides stimulated many investigators to develop new or to improve the old methods of analyzing for this element. Scott's *Standard Methods of Chemical Analysis*

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(295) revised in 1938 by Furman et al., and the review by Hernler and Pfeningberger (129) contain valuable detailed summaries of the various methods of analyzing for fluorine compounds.

At present, the method used most widely for the analysis of biological material is that of Willard and Winter (370) or some modification of it (5) (7) (42a) (52) (66) (112) (127) (188) (200) (205) (263) (264) (265) (266) (279) (293). The conflicting reports in some of the literature are due mainly to the fact that adequate methods were not available or the investigators were not aware of the many substances and experimental conditions which interfere with fluorine determinations. For example, excessive concentrations of sulfates and phosphates interfere with most methods used in the determination of fluorine. Fluorine compounds should be separated from the interfering substances or appropriate corrections made for their presence. In ashing samples for analysis, a fixative should be present (e.g., $Al(NO_3)_3$, CaO) and the mixture heated at a low temperature, not to exceed 600–650°C., because fluorine compounds are generally volatile above this temperature.

OCCURRENCE AND DISTRIBUTION. Fluorine is one of the most active elements chemically, consequently it does not occur in the free state in nature. The term "fluoride intoxication" has been used in this review to indicate fluorine in the combined form; however, not all fluorine compounds are fluorides technically. Fluorine compounds are frequently found in gases from volcanoes and fumaroles. Igneous rocks usually contain this element in varying amounts (0.01 to 3.36 per cent) and its compounds have been found widely distributed in many types of rock formation.

A. *Industrial uses of fluorides.* The principal fluorine bearing minerals which are used in large scale industrial processes are: fluorite or fluorspar (CaF_2), cryolite (Na_3AlF_6), apatite ($3Ca_3(PO_4)_2 \cdot CaF_2$) and sedimentary phosphate rock.

Fluorspar is used extensively as a flux in the smelting of many metals and in the ceramic industries. It has been estimated that 3 to 5 kgm. of it are used in the average production of one ton of steel (168) (273). During the smelting process, silicon tetrafluoride (SiF_4) is formed according to the following reaction: $3\ SiO_2 + 2\ CaF_2 \rightarrow SiF_4 + 2\ CaSiO_3$. The world production of fluorspar was about 525,000 metric tons in 1937 (4).

Fluorine compounds are used widely in glass, enamel and brick production.

Cryolite is used in the manufacture of aluminum by the Hall-Heroult electrical process which is employed extensively today. Reactive fluorine compounds are produced during the process. The total world production of aluminum was about 600,000 metric tons in 1938 (192).

Large deposits of phosphate rock are found in Colorado, Florida, Idaho, Montana, South Carolina, Tennessee, Utah, Wyoming and also in other parts of the United States and the world (139). Phosphate rock (containing about 3.5 per cent F) (133) is used in the manufacture of superphosphate (retaining approximately 75 per cent of the F) which is used in animal and plant nutrition. The world use (industry and agriculture) of apatite and phosphate rock was approximately 12,025,000 metric tons in 1938 (138).

While the above industrial processes represent the more important large scale sources of possible fluoride intoxication, table 1 contains a summary of other, small scale uses of fluorine compounds in technical processes which may also lead to fluorine ingestion by man. The data indicate that fluorine compounds are used widely in industrial processes.

B. *Occurrence of fluorides in edible foods.* Fluorine compounds are present in numerous foods. The amount varies with the type as well as with the sources of the foods. McClure (206) has prepared a summary of the fluorine content of foods and beverages reported in the literature (4) (50) (65) (77) (79) (92) (141) (180) (208) (220) (221) (263) (334) (375). The fluoride content of most edible foods ranges from traces to about 10 p.p.m.,[2] with foods containing 1–2 p.p.m. being most common. The foodstuffs containing the greatest amounts of fluorides are certain teas, phosphate baking powders, bones, fruits and vegetables which have been sprayed with fluorine-bearing insecticides and then not washed adequately. Gaud et al. (104) found that wheat and barley grains produced in normal areas in North Africa showed 2 and 3 p.p.m. of fluorine while such grains raised in fluorine-containing rock phosphate areas contained as much as 226 and 184 p.p.m. respectively. They estimated that at least three fourths of the fluorine found in these plants analyzed was due to the dust on the surface of the grain.

The fluorine content of most teas ranges from 9 to 399 p.p.m. Reid (260) analyzed one sample of fresh tea leaves (grown in a fluorite mining area in China) which contained 1757.8 p.p.m. of fluorine. He also found that from 81 to 96 per cent of the fluorine in tea leaves was ex-

[2] p.p.m. = parts per million.

tracted in a 2 per cent infusion. It is important to note that the ingestion of 1 and 2 per cent infusions of a tea, containing 399 p.p.m.

TABLE 1

Uses of fluorine compounds in technical processes

USES	COMPOUNDS
1. Binder for emery wheels	CaF_2 (273)
2. Bleaching of cane for chair seats	HF (273)
3. Bleacher in laundries	Na_2SiF_6 (273)
4. Cleansing graphite	HF (273)
5. Coagulating rubber	Na_2SiF_6; $MgSiF_6$ (273)
6. Decolorizing of glasses	Na_2SiF_6 (85)
7. Disinfection of hides and skins	H_2SiF_6 (334)
8. Disinfection of hose and tanks in breweries	H_2SiF_6; $NH_4F \cdot HF$ (273)
9. Electrolytic manufacturing of beryllium	Na_2SiF_6 (273)
10. Fixing of tannin on cotton in dye works and printing works	SbF_3 (273)
11. Flotation of fluorspar and quartz	CaF_2 (16) (218)
12. Flotation of lead-zinc-fluorspar ores	CaF_2 (55) (56) (80)
13. Flux	Na_3AlF_6; Na_2SiF_6; CaF_2 (361)
14. Glass etching	HF; NH_4F (273) (290) (340)
15. Hardening cement	$MgSiF_6$; $ZnSiF_6$ (273)
16. Insecticidal powders	NaF (142)
17. Making glue, paste and adhesives	HBF_4 (273)
18. Making synthetic stones	Sundry compounds (273)
19. Optical industry	CaF_2 (273)
20. Preserving wood (building) timber, telegraph poles	NaF; ZnF_2; $MgSiF_6$ (44) (62) (136) (137)
21. Recovery from phosphate plant	H_2SiF_6; $PbSiF_6$ (228)
22. Refining mineral oils	SO_2F_2; SF_6 (342)
23. Refrigerating industry	CCl_2F_2; $C_2Cl_2F_4$ (34) (270) (379) (273)
24. Removing sand from castings	HF (273)
25. Rust removal from steel and iron	HF (273)
26. Silicate analysis and condensing agent in laboratory	HF
27. Silk dyeing	HF (273)
28. Synthetic production of cryolite	CaF_2 (177) (347)
29. Treating anthracite for making gas coal	HF (273)
30. Window cleaner	H_2SiF_6 (273)
31. Wood-staining	CrF_3 (273)

of fluorine in the dry substance, causes tooth striations in the rat in four to five weeks. Reid and Cheng (261) found that the feeding of

fluorine present in 1 and 2 per cent infusions to female rats during pregnancy and lactation resulted in an increased storage of fluorine in young rats at weaning. The permeability of the placenta to fluorides has been reported (154) (235) (354).

Phosphate baking powders (206) contain from 19 to 220 p.p.m. of fluorine and edible cooked bone (206) 93 to 367 p.p.m. In the rat, naturally occurring fluorine compounds in canned salmon and mackerel (169) was stored at about one-third the rate of added inorganic fluorides. The latter was also three times as effective in producing a bleaching of the enamel as the former.

Fluorine-containing insecticides such as cryolite (impure Na_3AlF_6) and barium fluosilicate ($BaSiF_6$) are now being employed as sprays for fruits and vegetables (198) (380). Fluoroarsenates of zinc, copper, aluminum and magnesium have been recommended as insecticides recently (118). Unless these substances are removed from these foods, they constitute an additional fluoride hazard (119) (314). Carter and Busbey (45) have reviewed the literature relating to fluorine in insecticides and related compounds. These sprays are replacing lead arsenate in many areas. Overley (239) reports that 1,786,000 lbs. of cryolite fruit-spray were used in the state of Washington in 1938, and estimated that the amount used in that state in 1939 would be slightly less than in 1938. The amount of fluorine found on unwashed apples varied from 0.15 to 0.40 grain per pound of fruit (21 to 57 p.p.m.) depending upon the number of cover sprays and other factors. Most of the fluorine residue can be removed from the sprayed fruit by brushing in a dilute hydrochloric acid solution and washing with water.

McClure (206) has compiled data on the fluoride content of baby foods. The fluorine content of such foods should be kept at a low level, particularly during the period of calcification of the teeth (276).

C. *Occurrence of fluorides in drinking water and its correlation with mottled enamel.* Smith (308) has determined the fluoride content of 55 Arizona water supplies by the methods of Fairchild (99), Foster (100), Sanchis (281), and Willard and Winter (370). These methods have been used frequently to determine the fluoride content of water. The results obtained by the Foster, Sanchis, and Willard and Winter methods were in close agreement, while the Fairchild method gave results which averaged two to three times higher on certain waters than the other three methods. Mottled enamel was associated with a fluoride concentration of 0.9 to 1.0 p.p.m. or greater in Arizona waters (Foster, Sanchis, and Willard and Winter methods). Data from other areas (53) (72) reveal that the consumption of water containing

1 p.p.m. or more of fluoride (analyzed by Foster, Sanchis, Willard and Winter or equivalent method) during the period of calcification of the teeth results in the production of mottled enamel in man.

Mottled enamel and waters containing fluorides in concentrations of 1 p.p.m. or above occur in many areas in the United States (1) (10) (12) (26) (30) (52a) (53) (57) (68) (72) (76) (101) (111) (135) (143) (144) (158) (161) (173) (190) (237) (238) (258) (294) (307) (311) (319) (351) (364) (368) (371). Drinking waters containing 1 p.p.m. or more of fluoride appear to be most prevalent in the middle west and southwestern parts of the United States. While accurate data are not available regarding the number of people who are consuming water containing toxic concentrations of fluoride, estimates from Illinois (368) and Texas (17) indicate that about 330,000 and 500,000 persons respectively are involved in these two states alone.

Mottled enamel and waters containing toxic amounts of fluoride have been reported from Africa (103) (277) (324) (325) (331), Asia (47) (225) (236) (257) (300) (301) (335) (336) (367) (372), Europe (3) (32) (48a) (227) (268) (329) (372), South America (67) 86) (233) (344) (345) (346), and other parts of the world (54) (176) (277) (365).

FLUORIDE INTOXICATION IN MAN. A. *Local action.* Hydrofluoric and hydrofluosilic acids and their more soluble salts have a corrosive effect on living membranes. Brief exposure causes redness and a prolonged burning sensation. Longer exposures or more concentrated solutions produce yellowish, coriaceous changes in tissues which later develop into painful and slow-healing ulcers (282). Seldom is such injury permanent although a few cases have been reported with secondary fatal termination.

Gaseous silicon tetrafluoride and hydrogen fluoride are extremely irritating to the conjunctiva and mucous membranes of the respiratory passages, causing sneezing, coughing, hoarseness and increased secretions from nose and eyes. Among workers exposed to dust containing fluorine compounds, acute pulmonary symptoms such as bronchitis, dyspnea, and asthma have been reported (88) (277).

B. *Acute intoxication.* Roholm (273) has prepared a detailed summary of the acute cases of fluorine intoxication which have been reported in the literature from 1873 to 1935 inclusive. During the period 112 cases were described, 60 of which ended fatally. Several additional cases (19) (41) (105) (129a) (376) have been observed since 1935. In most instances, these were the result of accidents in which fluorine-containing insecticides, rat poison or other preparations were

ingested in large quantities by mistake. Sodium fluoride, sodium fluosilicate, hydrofluoric acid and hydrofluosilicic acid, in the order named, were the compounds most frequently involved. The cases described by Griebel et al. (116) are of special interest because mass illness was traceable to apricot preserves containing excessive fluorine. Roholm (272) has made an analysis of the mysterious fog disaster which occurred in the Meuse Valley near Liege, Belgium in December of 1930. He states that the circumstantial evidence indicates that the disaster was probably due to acute fluorine poisoning which resulted from gaseous fluorine compounds (HF, SiF_4) liberated by certain factories in the area in conjunction with an unusually heavy fog. Several thousand persons suffered acute pulmonary attacks, and 60 deaths were reported.

The symptoms of acute intoxication are partly local, affecting the gastro-intestinal tract (thirst, vomiting, abdominal pains, diarrhea) and partly due to absorption which results in alternate painful spasms and pareses, weakness, excessive salivation, perspiration, dyspnea and weakened pulse. Death is attributed primarily to respiratory paralysis. One or more of the symptoms may be absent. Post-mortem examinations reveal a marked hemorrhagic gastro-enteritis with a tendency to necrosis. Microscopic findings have indicated a more or less pronounced degeneration in the parenchymatous organs (particularly the liver and kidneys).

With the exception of a few cases, where the fatal dose was as low as 0.2 and 0.7 gram of sodium fluosilicate for adults, the lethal dose ranged from 5 to 15 grams of sodium fluoride or sodium fluosilicate.

C. *Chronic intoxication.* The first detectable symptom of chronic fluorine poisoning is a hypoplasia of the teeth which has been called mottled enamel, darmous and gaddur. The condition consists of an irregular distribution of grayish white blotches or chalky areas over the entire tooth surface and involving all teeth. Upon some teeth there is an irregular distribution of pits; on others, an incomplete calcification of the cusp tips, and in extreme cases, the entire tooth surface is pitted. This general condition is accentuated in many cases by a discoloration ranging from light brown to almost black. The permanent teeth are affected most frequently; however, mottled deciduous teeth have been reported (313) (322). The damage occurs during the process of calcification of the teeth. In man, this process, with the exception of the third molars, occurs during the first eight to nine years of life.

The details of the macroscopic and microscopic changes occurring

during dental fluorosis have been described (6) (35) (38) (69) (74) (87) (149) (152) (173) (226) (234) (283) (283a) (284) (286) (287) (288) (314) (364). Schour and Smith (285) believe "that fluorine exerts a direct local action on enamel forming cells and that the changes observed in the enamel and dentin are not produced primarily by changes in blood calcium or phosphorus or by disturbances in the parathyroids."

Roholm et al. (278) observed no significant variation in blood calcium or inorganic phosphorus in cryolite workers with varying degrees of osteosclerosis. Normal values for blood calcium, inorganic phosphorus, hemoglobin and coagulation time were found in puppies which had received fluorine at levels (0.45–4.52 mgm. per kgm. of body weight) comparable to and higher than that consumed by man in some mottled enamel areas, during the eruption of the permanent teeth (113) (21). Blood sugar and nitrogenous constituents were within normal limits in dogs fed 4.5 to 13.6 mgm. of fluorine (as NaF) per kgm. of body weight for 4½ to 12 months (115). Large doses of fluorides, however, produce changes in the blood constituents (61) (151c) (262) (337). Fluorine compounds have been reported to alter the structure and function of the parathyroids (19a) (45a) (155) (239a). On the other hand, other investigators have not been able to find any consistent gross or microscopic changes in the parathyroids with the administration of toxic doses of fluorine compounds (123) (124) (125) (151c).

The minimum amount of fluorine required to produce mottled enamel has not been determined accurately. DeEds (72) estimated that the daily intake of 0.1 to 0.15 mgm. of fluorine per kgm. of body weight in the drinking water is sufficient to produce mottled enamel. This calculation does not include the fluorine content of the food. McClure (206) has calculated that an average child of 15 kgm. living in a mottled enamel area where the water contained 2 to 3 p.p.m. of fluorine would consume 0.15 to 0.30 mgm. of fluorine per kgm. of body weight daily. Machle et al. (182) (185), studying the relation of urinary fluorine excretion to the fluorine content of the food and water ingested, concluded that: 1. The fluorine content of foods appears to play a minor rôle in the production of mottled enamel; however, "amounts equivalent to or slightly greater than those encountered in foods grown in endemic areas are not associated with the production of mottled enamel where there is not an accompanying elevation in the fluorine content of the drinking water." 2. Analyses of samples of urine from people living in different parts of the United States where the fluorine content of the water is less than 1 p.p.m. revealed that there is a normal urinary

fluorine excretion of approximately 1 mgm. per liter of urine. 3. There appears to be a direct correlation between the high fluorine content of the drinking water and the amount of fluorine excreted in the urine. Where mottled enamel is endemic and the drinking water is high in fluorine, the urinary excretion of fluorine is much greater than in areas having no mottled teeth. Smith (323) reports 24 cases of mottled enamel in an area where the fluoride content of the water was less than 1.0 p.p.m. Most of these patients had febrile diseases during childhood.

Shortt et al. (300) have made detailed clinical, radiological and biochemical investigations of ten chronic cases of fluorine intoxication from a mottled enamel area in India. The people consumed the high fluoride water for 30 to 45 years before clinical symptoms of bone changes and possible kidney impairment occurred. The clinical picture related essentially to disabilities caused by calcification of ligaments, tendons, fasciae, the formation of osteophytic outgrowths of bones and nervous effects of mechanical pressure due to the encroachment of bone on the spinal cord. The radiological evidence also revealed excessive calcification of tendons, ligaments and fasciae, the production of osteophytic formations on various bones, and almost complete synostosis of the various joints, especially of the vertebral column. The radiographs provided the clues to the clinical observations. Serum calcium, inorganic phosphorus and phosphatase indicated a favorable condition for abnormal deposition of bone. In the majority of cases kidney function was also impaired.

Blue (24) has made a survey of the general physical development of children living in normal and endemic mottled enamel areas in the Panhandle of Oklahoma. He states that the general development of the children, as indicated by the number with fractured bones, rickets and dental deformities, is retarded in the mottled enamel areas where the drinking water contains more than 1 p.p.m. of fluorine. This study was based on clinical observations and no data were reported regarding the intake of essential constituents of the diet, such as calcium, phosphorus and vitamins concerned with bone and tooth formation and maintenance. Calcium and phosphorus balance studies on girls living in mottled enamel areas in Arizona revealed a normal assimilation of these elements (165) (319). When young rats were fed 0.1 per cent sodium fluoride "their ability to metabolize calcium and phosphorus was decidedly impaired" (164).

Human osteosclerosis has been found to be due to the ingestion of

fluorine compounds for several years. This condition can be identified radiologically and it was first described by Møller and Gudjonsson (224) and Møller (223) among cryolite workers. Roholm (273) has made a more comprehensive study of the cryolite workers in Denmark and estimated that they regularly ingested from 0.20 to 0.35 mgm. of fluorine per kgm. of body weight daily. He has described the essential changes briefly as follows: "The first thing to emphasize is the fact that the affection is a system-disease, for it attacks all bones, though it has a predilection for certain places. The pathological process may be characterized as a diffuse osteosclerosis, in which the pathological formation of bone starts both in periosteum and in endosteum. Capacta densifies and thickens; the spongiosa trabeculae thicken and fuse together. The medullary cavity decreases in diameter. There is considerable new-formation of bone from periosteum, and ligaments that do not calcify or only in advanced age undergo a considerable degree of calcification. All signs of bone destruction are absent from the picture." The histopathology of the bone changes in chronic fluorosis have been described (145) (163) (199) (338). Robison and Rosenheim (269) have studied the calcification of hypertrophic cartilage of rat in vitro. They found that sodium fluoride in concentrations as low as 0.00001 M prevented calcification of slices of rat cartilage in solutions containing 8 mgm. of calcium and 5 mgm. of inorganic phosphorus per 100 cc. of solution.

Speder et al. (324) (325) and Gaud et al. (104) have found similar changes occurring in man in "phosphate zones" containing considerable concentrations of fluorine (water, soil, dust) in North Africa. Bishop (22), Bauer et al. (15) and Wolff and Kerr (377) reported a case of osteosclerosis occurring in a man who had worked in a phosphate fertilizer plant in the United States. More recently, Capizzano et al. (38a) have observed bone changes in endemic mottled enamel areas in La Pampa, Argentina. Although the number of cases studied has been limited, the findings are typical of fluorine poisoning.

Many studies have been reported concerning the influence of fluorine compounds on the thyroid (82) (96) (108) (109) (110) (134) (203) (222) (297) (324) (324a) (333) but there is no general agreement regarding how the gland is affected. Goldemberg (107a) reported beneficial results from the use of sodium fluoride in the treatment of hyperthyroidism. Phillips et al. (245) (247) (248) have not been able to confirm the results of Goldemberg on rats, guinea pigs or chickens. Litzka (179) found that 3-fluorotyrosine acted antagonistically to thyroid

secretions. Kraft (156) observed that this compound retarded the increased rate of metamorphosis of tadpoles induced by the addition of thyroxine. May (202) treated hyperthyroidism cases with 3-fluorotyrosine and noted a decrease in the size of the neck and basal metabolic rate. Kraft and May (157) determined the fluorine content of the whole blood of normal persons and one with hyperthyroidism. The former contained 0.105 and the latter 0.062 mgm. per cent of fluorine. The blood serum of the hyperthyroid patient contained 0.080 mgm. per cent of fluorine; however, after five months' treatment with 3-fluorotyrosine it was increased to 0.117 mgm. per cent. May and Litzka (201) have reported that 3-fluorotyrosine inhibits the growth of certain tumors.

Evans and Phillips (89) have determined the fluorine and iodine content of the thyroid glands from about forty patients with varying degrees of hyperthyroidism who had undergone thyroidectomies. The basal metabolic rate was determined before and after the operation on each patient. In general, no correlation was observed between the fluorine content of the thyroid gland and the basal metabolic rate of the patient. In the majority of cases, no relationship was noted between the iodine and fluorine content of these glands; however, "in two cases in which double lobectomies were performed, a decrease in the fluorine content of the thyroid occurred in the interim between the two operations. This was accompanied by a decrease in the basal metabolic rate and an increase in the iodine content of the gland." Further studies are necessary to determine the effects of fluorine compounds on the structure and function of the thyroid.

INTOXICATION IN ANIMALS. The literature relating to acute and chronic fluorine poisoning in animals has been summarized by Roholm (273) and Peirce (241), hence only a brief résumé will be given in this review.

A. *Acute* fluorine poisoning has been observed in nearly all of the common animals and the symptoms and pathological reports are similar to those recorded for man, except that inflammatory changes in the kidney have been described more frequently in the case of animals. Adequate quantitative data regarding the fatal dose for the various species are not available. There is considerable variation in the acute toxic dose of the different fluorine compounds (36) (37) (114) (277) (303). In general, the more soluble alkali fluorine compounds are the most toxic.

B. *Chronic.* The literature is replete with reports of the effects of

spontaneous and experimentally produced chronic fluorine intoxication in animals (58a) (122a) (153) (160) (164) (170) (175) (178) (181) (183) (184) (186) (271) (278a) (289) (289a) (302) (304) (306) (312) (316) (317) (320) (330) (338) (341) (374). These investigations have been concerned primarily with three types of problems:

1. The effects of the use of rock phosphate, or similar materials, which contain excessive fluorine as calcium and phosphorus supplements in the ration of animals or as soil fertilizers have been studied by many investigators (20) (46) (48) (49) (81) (83) (90) (94) (95) (121) (123) (124) (126) (128) (132) (162) (172) (229) (236a) (244) (246) (249) (250) (254) (255) (267) (291) (325) (326) (327) (328). The most complete recent researches on this phase of the subject have been reported by Du Toit et al. (78), Gaud et al. (104), Kick et al. (151a, b, c), Peirce (240), Phillips et al. (243–256), Schulz (292), and Velu et al. (352–360). The data indicate that the typical symptoms of chronic fluorine poisoning observed in man develop in the animals. Since the animals have generally received larger doses of fluorine than man, more severe symptoms, such as bone and kidney damage, have been reported more frequently.

Du Toit et al. (78) have reported fluorine metabolism studies in rats and bovines in which various concentrations (60 and 738 mgm. F daily) of calcium fluoride (CaF_2) and mono-calcium phosphate ($CaHPO_4$) were used as the source of fluorine. They found that fluorine ingested in these concentrations caused a decreased retention of both calcium and phosphorus in rats, and slight increase in calcium retention and a pronounced decrease in the retention of phosphorus in bovines. Kick et al. (151c) found that when the ration of pigs contained more than 290 p.p.m. of fluorine as sodium fluoride or more than 330 p.p.m. as rock phosphate, the bones were characterized by increased thickness, loss of normal color, presence of exostoses, and decreased breaking strength. They also observed that the addition of 1 per cent of rock phosphate (containing about 3.5 per cent F) to the rations of pigs caused a degeneration of the epithelium of the convoluted tubules and a fibrosis of the kidney. This did not occur in the case of the rat or pigs fed similar or higher levels of sodium fluoride, calcium fluoride and phosphatic limestone.

Peirce (240) fed sheep various levels (60–170 mgm. daily) of fluorine in the form of rock phosphate for a period of three years. All of the animals appeared to be in good health during the first year. Thereafter, some of them began to eat less and lose weight. The bones of animals which received doses greater than 120 mgm. of fluorine daily

exhibited the bone changes described by Kick et al. (151c). No organs, except the bones and teeth, appeared to be affected by the treatment.

Phillips et al. (251) have made long continued detailed investigations of the effect of adding various levels of rock phosphate (containing approximately 3.55 per cent F) to the rations of dairy cows, chickens, guinea pigs, rats and swine. Their experiments with dairy cows indicated that 200 to 880 p.p.m. of fluorine in the ration exerted little detrimental effect upon growth during the first two years of the feeding period; however, a decrease in body weight occurred later, from which the animals did not recover. Milk production decreased significantly when the higher levels of fluorine were fed. Chemical and biological tests revealed that no significant changes in the nutritional qualities of the milk occurred (252). The gross symptoms and pathology observed at autopsy of the cows showed the typical changes of fluorosis (253).

The gross and microscopic pathological data and supplementary oxygen uptake studies on certain tissue induced these investigators to suggest "that fluorine toxicosis produces its systemic reaction through an interference with cellular respiration and that the primary point of attack is the enzymatic systems of the body" (251) (256). Phillips (243) has furnished a summary of his researches relative to the tolerance of the different species to the fluorine found in rock phosphate. The milligrams of fluorine per kilo of body weight required to affect growth were as follows: bovine, 1–2; swine, 10–12; rat 18–20; guinea pig 20–25; chicken 35–70. He has found that fluorine in rock phosphate and in calcium fluoride is roughly half as toxic as sodium fluoride. Growth was used as a criterion of toxicity.

Schulz (292) has investigated the toxicity of different fluorine compounds and fluorine bearing mineral supplements (which are fed to farm animals) in various diets in the rat. He found that the calcium, phosphorus and cod liver oil content of the ration had an ameliorating effect upon the fluorosis, when certain diets were fed. He concluded that "no levels are suggested at which earthy phosphate may be safely fed to farm animals."

Gaud et al. (104) and Velu and Charnot (360) have made extensive studies of fluorosis (darmous) in sheep and other animals in the phosphate zones of northern Africa. They consider the important factors responsible for the intoxication are: the fluorine of the suspended matter in the water, the increased fluorine content of plants growing in phosphate regions, and especially the fluorine in the dust on the plants.

Hart et al. (122) have studied the relation of soil fertilization with

superphosphate and rock phosphate to fluorine content of plants and drainage waters. They collected data from several agricultural experiment stations in the United States where fertilization with fluorine bearing mineral supplements had been practiced for many years. They found that "plants from plots treated with fluorine containing rock phosphate and acid phosphate, for periods of 16 to 36 years, did not show consistent or greatly increased fluorine content over plants produced in soil receiving low fluorine fertilizer such as bone meal." The fluorine content of drainage water from a single series of lysimeters in Tennessee "showed a particularly high content where the soil had been treated with rock phosphate, and also a definitely increased fluorine content when treated with superphosphate as compared with the control." These investigators concluded that: "With our limited available data, nothing further is intended in this article than to call attention of public health officials, agronomists, and fertilizer manufacturers to the problem that confronts them in the present practice of adding fluorine to our soils."

Bartholomew (14) has reported on the effect of fluorine in the soil on plant growth and composition. In general, he found no significant changes either in the composition or growth of plants on the soil treated with fluorine containing materials. MacIntire et al. (189) have studied soil treated with 1500 pounds of barium fluosilicate per acre. They found that the fluorine united with calcium in the soil system, except where either magnesic or dolomitic incorporations were made. MacIntire (187a) has investigated the influence of the additions of calcium fluoride, as such, in the precipitated form, and as a component of either phosphatic fertilizers or by-product calcium silicate slag, containing about 3½ per cent of fluorine. He has added up to 4500 pounds of calcium fluoride per acre. He states: "In general, we have found that additive fluorides do not bring about any enhancement in the fluorine content of the tops of plants. We do find, however, an appreciable concentration in the fluorine content of roots of red clover and sudan grass and possibly the same in one or two other crops.... Concentration of fluorides does not take place, however, in tap roots, such as carrots....We have much to learn in regard to the fate of fluorides supplied to the soil through rain waters[3] and through fertilizer treatments and as an incident to fluoride insecticides. We believe that we should expect considerable variation for *different types of soil of*

[3] Leachings from fluoride treated soils.

variant fixation capacity and of different content of calcium due to either native compounds or added liming materials."

Carlson (39) has raised the following question, which should be given careful study: "What is going to be the condition 25, 50 or 100 years hence on our farms if we continue with fluorine bearing rock phosphate fertilizer, and continue with lead, arsenic and fluorine sprays?" He has also suggested that "a long range scientific investigation should be initiated before we seriously damage our soil and unwittingly add poisons to our diets in water, grains and fruits." There are already areas in the United States where the soil has been poisoned with arsenic and lead to the extent that plants will not grow on them. Soils need calcium and phosphorus supplements yet these important elements can be supplied without the addition of excessive fluorine or other toxic constituents which may eventually be harmful.

2. Fluorine bearing insecticides are now being used as sprays for fruits and vegetables. Cryolite (Na_3AlF_6) and barium fluosilicate ($BaSiF_6$) are the compounds which are used most widely. Marcovitch (193) has suggested the use of magnesium oxide as a "corrective" in cryolite sprays. Detailed information relating to insecticides is contained in the review by Carter and Busbey (45).

The United States Department of Agriculture has set a maximum limit for marketable sprayed produce as 0.02 grain of fluorine per pound (2.8 p.p.m.). This tolerance applies only in interstate commerce. The fluorine content of foods should remain low until more adequate data are available regarding the chronic effects of the ingestion of small amounts of this element.

Animal experiments relating to the toxicity of fluorine bearing insecticides have been conducted by numerous investigators. Marcovitch et al. (194) (196) found that when fluorine compounds were fed as powders in the whole diet, the lowest levels which caused striations in the tooth enamel in some of the rats, in p.p.m. fluorine, were cryolite 7, sodium fluoride 7, and calcium fluoride 15. They also observed that water containing 1 or 2 p.p.m. of fluorine, as sodium fluoride, when used for both drinking and cooking produced striations in incisor teeth of rats. Marcovitch and Stanley (195) made a comparison of sodium fluoride in the drinking water with similar levels of cryolite in the diet on the fluorine content of the body in the rat. They concluded that 4 p.p.m. of fluorine in drinking water, as sodium fluoride, caused a storage of nearly twice as much fluorine as 4 p.p.m. cryolite in the diet.

Some of the experimental methods and interpretations by Marcovitch

et al. are not shared by Lawrenz et al. (166) who have made a comparative study of the retention of fluorine as calcium fluoride and cryolite, when both were administered in aqueous solution at the rate of 0.58 mgm. per kgm. of body weight daily in the diets of rats. They observed the appearance of striations in the incisor teeth occurred at about the same time with both compounds and also that the retention of fluorine was about the same (59.2 and 59.3 per cent). Approximately 96 per cent of the fluorine retained at this level of feeding was deposited in the skeleton and the remaining 4 per cent was about equally divided between teeth and soft tissues.

Lawrenz et al. (167) also investigated the toxicity of fluorine in the form of cryolite when fed in the water and in the food at a concentration of 10 p.p.m. They found that the method of administration at low levels of intake had no apparent effect on the rate of growth of the animals. The addition of fluorine to the water, however, definitely depressed the appetite of rats and induced a transient hematuria at the beginning of the experiments. Fluorine in the food was retained to a less extent (20 per cent) in the body than equal doses in the drinking water. This was explained on the basis of impairment in absorption from the alimentary tract. They concluded that "considering both the difference in the usual consumption of food and water in practical human nutrition, and the difference in potential toxicity of fluorine in water and in food, a concentration of 1 p.p.m. of fluorine in the drinking water defining the upper limit of safety, is the hygienic equivalent of from 2.4 to 4.8 p.p.m. of fluorine in the total food, depending upon the proportion of the water intake that contains fluorine in the critical concentration."

Evans and Phillips (91) have determined the comparative toxicity of the fluorine in the form of cryolite and sodium fluoride in the rat. They found that 4 p.p.m. of fluorine added to the drinking water as sodium fluoride or as cryolite resulted in similar storage of fluorine in the bones of the growing rat. At higher levels (600 p.p.m.) less fluorine (about half) was found in the skeleton when it was fed as cryolite or as sodium fluoride-aluminum chloride mixture than when it was fed as sodium fluoride.

3. Chronic fluorine poisoning has been observed in the form of a disease similar to osteomalacia in livestock which had grazed in the vicinity of aluminum and super-phosphate factories. This condition has been reported in Denmark, France, Germany, Switzerland (272) and Italy (13) (197).

Slagsvold (305) has given a detailed description of this manifestation of fluorosis occurring in sheep and cattle which had grazed near Norwegian aluminum works. Many of the typical symptoms of fluorine poisoning (listed previously) were observed. The back and limbs of the animals became tender and stiff. Bone fractures were common but it was doubtful if thickening of the bones occurred. The vegetation grown in these areas showed an increased fluorine content. It was possible to demonstrate experimentally that fluorine compounds produced the symptoms which spontaneously developed in the cattle grazing near the aluminum factories.

Bredemann and Radeloff (33) have reported the absorption of fluorine from smoke by certain plants.

The relation between the symptoms of chronic intoxication and the dosage of fluorine in rats has been tabulated by Roholm (273) as follows:

SYMPTOMS	F PER KGM. OF BODY WEIGHT PER DAY
	mgm.
Incipient tooth changes	1
Incipient bone changes and nephritis	5
First effects on general condition	10–15
Severe influence on general condition, organ degeneration	20–25
Death in one or few weeks	50–100

He emphasized that the doses in this summary were only approximations and future researches will be required to establish accurate limits. There is a significant variation in the toxicity of different fluorine compounds (75) (106) (110) (114) (148) (298) (321) which should be considered in establishing reliable limits. Evans and Phillips (243) have found the toxic level of 3-fluorotyrosine, 3-fluorophenylalanine and 3-fluoro-5-iodotyrosine in the ration of rats to be 0.5, 1.0 and 1.0 mgm. per kgm. of body weight respectively. The severity of the tooth, bone and other damage depends upon the type of fluorine compound, dosage, duration of exposure and the intake of other essential constituents of the diet, such as calcium and phosphorus and vitamins (A, D and C) involved in the formation and maintenance of these structures.

The effect of fluorides on the activity of the brain has been studied (231) (232) (242).

Wilson and DeEds (373) have reported that the feeding of small concentrations (31 to 500 p.p.m.) of cadmium as cadmium chloride in the diet of rats causes a bleaching of the teeth "similar to, if not identical

with that produced by fluorides." Anemia and cardiac hypertrophy were also observed in these rats.

Does fluorine have a physiological rôle in the animal kingdom? Several investigators have dealt with the possible rôle of fluorine in the animal organism (216) (259). Gautier (102) postulated that fluorine had some bearing on the binding of phosphorus in the cell and that it contributed to the hardness and resistance of certain tissues (teeth, feathers, etc.) to various injurious chemicals. More recently, Armstrong et al. (8) (9) and others (59) (60) (70) (71a) (131) (217) have demonstrated that fluorides appear to decrease the incidence of dental caries in man and experimental animals. On the other hand, McCollum and Sharpless (299) have fed rats on a diet low in fluorine for three generations. The animals appeared to be normal, and the bones and teeth contained extremely small quantities of fluorine. Evans and Phillips (93) fed rats for five generations on a milk ration, supplemented with essential minerals and vitamins. The basal ration contained 0.1 to 0.2 p.p.m. of fluorine. The animals appeared to be normal and the skeletal storage of fluorine was extremely low (8 to 16 p.p.m.).

Carlson (40) has suggested that the addition of small quantities of fluorides to tissue cultures of bone and other tissues might yield valuable data regarding the possible function of fluorine compounds in physiological processes. According to Phemister (242a), the action of fluorine on bone is primarily in the nature of irritation.

Recent reports by Dean et al. (70) (71a) have revealed that the incidence of dental caries is less in people living in mottled enamel areas where the water contains 1 p.p.m. or more of fluoride than in areas where the fluoride content is low. They associated the low caries rates with the presence of small amounts of fluorides in the water, although they suggest that the composition of the water in other respects may be a factor requiring further investigation. Blumberg et al. (25) and Lowater and Murray (181a) analyzed the teeth of rats fed diets containing fluorine and found that the fluorine content of the teeth increased. Boissevain and Drea (27) found that the fluorine content of bones, enamel and dentin of people who had lived in mottled enamel areas in Colorado (fluorine content of water was about 2 p.p.m.) for at least ten years was significantly higher than similar tissues from areas where the fluorine content of the water was low and mottled enamel did not occur.

Armstrong (8) and later Armstrong and Brekhus (9) reported that

the enamel and dentin of sound human teeth had a mean fluorine content of 111 and 169 p.p.m. respectively. The fluorine content of the enamel of mottled human teeth varied from 245 to 361 p.p.m. and the dentin contained 371 to 425 p.p.m. They also found the mean fluorine content of sound and carious teeth was 111 and 69 p.p.m. respectively. They suggested further a possible relationship between the fluorine content of enamel and resistance to dental caries. Miller (217) has found that the addition of sodium fluoride and iodoacetic acid to a caries-producing diet fed to rats diminished greatly the incidence of carious lesions in the molar teeth.

Cox et al. (59a) (60) and others (131) (362) (363) have recently published data which confirm the findings of Miller. The concentrations of fluorine used in these studies were higher than the concentrations of fluorine found in most mottled enamel areas. Cox (59) has suggested that small quantities of fluorides be added to drinking water as a means of reducing the incidence of dental caries. According to Blayney (23), such a procedure should be discouraged at the present time because our knowledge regarding the chronic effects of the ingestion of small amounts of fluorides over several years' time under normal and pathological conditions is incomplete. Recent reports (38a) (104) (300), which have been discussed in another part of this paper, indicate that pathological bone changes are occurring in people living in endemic mottled enamel areas for long periods of time.

Effects on protoplasm and enzymes. Fluorides are general protoplasmic poisons. Roholm (273) (277) has summarized the literature relating to this phase of the subject. Fluorides appear to modify the metabolism of cells by changing the permeability of the cell membranes and by inhibiting certain enzyme systems. The exact mechanisms of such actions are still somewhat obscure.

Numerous investigators (47a) (120) (171) (174) (191) (219) (230) have used fluorides in their studies of dissimilation processes in muscle, yeast (280) and bacteria (369a). Fluorides interfere with the formation of pyruvic acid during muscle metabolism (213) (214) (215). Phillips et al. (251) have reported that blood of cows with chronic fluorosis showed an increase in blood phosphatase. Shortt et al. (300) have reported similar findings in chronic fluorine poisoning in man; however, the increase was not so marked. On the other hand, Smith and Lantz (318), Thomas et al. (343) and Roholm et al. (278) did not observe a significant increase in serum phosphatase in the rats or man with chronic fluorosis. Sodium fluoride at a concentration of 10 mgm. per

ml. of blood interfered with the determination of blood phosphatase unless it was removed before the determination was made (60a). The recent report by McClure (207) regarding the effect of various fluorides on salivary amylase revealed that relatively high concentrations were required to inhibit the action of this enzyme. Such data indicate that more quantitative studies should be conducted on the effects of fluorides on enzymes.

PROPHYLACTIC MEASURES. A. *Changing water supply.* The solution of the mottled enamel problem by changing the water supply has been greatly advanced by McKay (209) (210) (211). Kempf and McKay (150) suspected that some constituent of the water supply was involved in the production of mottled enamel and also noted that this condition was halted by a change in water supply in a city in Italy. In 1925, McKay examined the school children in Oakley, Idaho, and found mottled enamel endemic in the community. He recommended that the city change its water supply in a deliberate attempt to prevent mottled enamel. This was done, thus providing convincing evidence that mottled enamel could be prevented by this means. A subsequent analysis of the old Oakley water supply (prior to 1925) indicated 6 p.p.m. of fluorine, while the new supply contained less than 0.5 p.p.m. This unique experiment was started before the presence of excessive fluoride in the water supply was established as the cause of mottled enamel by Smith et al. (309) (315), Churchill (51), and Velu (352), and confirmed by others (238) (296).

Dean and McKay (71b) have recently reported that mottled enamel has been halted at Bauxite, Arkansas and Andover, South Dakota, by simply changing the common water supply from one containing excessive fluoride to one containing less than 1 p.p.m. The author is also aware of two other communities where mottled enamel has been prevented by substituting water supplies containing small quantities of fluoride for ones containing excessive amounts, confirming the observations of Dean and McKay.

B. *Removal of fluorine from drinking water.* The removal of fluorides from drinking water has been the objective of several recent investigations. Table 2 contains a brief summary of the methods used.

From a practical standpoint, alum, lime, certain forms of calcium phosphate and magnesium compounds are used most frequently in the removal of fluorides from drinking water. It should be realized that the composition of a given water, the type of treatment to which it might be subjected without regard to fluoride content, sources and cost

of raw materials, will determine which substance or substances would be most desirable in a specific case.[4]

C. *Removal of fluoride sprays from fruits and vegetables.* Excessive fluorine spray residue can be removed from fruits and vegetables by washing such products in a dilute acid (HCl or H_2SO_4) solution in the presence of aluminum, iron or boron compounds (42) (43) (45) (117).

D. *Decreasing fluorine content of feeds and fertilizer.* The removal of excessive fluorine from rock phosphate, which is used as a source of calcium and phosphorus for animals and plants, has been investigated (11) (28) (58) (63) (64) (130) (187) (198). The excessive fluorine is usually removed by heating the rock phosphate or other material to the temperature at which the fluorine volatilizes. Difficulties have been encountered in attempts to produce defluorinated phosphate on a commercial scale and the process is still in the development stage.

TABLE 2

Substances used to remove fluorides from drinking water

SUBSTANCES	REFERENCES
Alumina (activated)	(29) (51a) (97) (339)
Aluminum oxalate	(366)
Aluminum phosphate	(366)
Aluminum sulfate (alum)	(29) (31) (98) (146) (147) (148) (237) (294)
Bauxite (aluminum oxide)	(29)
Bone meal (treated)	(53) (310)
Carbon (activated)	(212)
Fused mixture of silicic acid, ferric chloride and barium chloride	(349)
Lime	(29) (378)
Magnesium compounds	(84) (366) (378)
Metallic oxide gels	(241a) (366)
Sand	(159)
Superphosphate	(350)
Titanium sulfate	(348)
Tricalcium phosphate and related compounds	(2) (18) (84) (187b)
Zeolites	(29) (107)

[4] The International Filter Company and the National Aluminate Corporation, both of Chicago, and there are undoubtedly others, are now manufacturing equipment and materials for the removal of fluorides from public and private water supplies.

At the present time the Tennessee Valley Authority is investigating suitable methods for the quantity production of this material (140) (187a).

SUMMARY

Fluorine compounds are widely distributed in nature and they are used in numerous industrial processes which result in fluoride ingestion by man and animals. The possible rôle of fluorine in normal physiological process is still obscure. Fluorides are general protoplasmic poisons in relatively low concentrations and they inhibit many enzymatic processes.

Acute intoxication. The following symptoms are observed when a single dose is taken by mouth: salivation, nausea, vomiting, urination, muscular weakness, excitement and tremors, convulsions and fall of blood pressure. At first there is an acceleration and deepening of the respiration which is followed by a general paralysis of the vital nervous centers. One or more of the symptoms may be absent. Death is due primarily to an inhibition of respiration. The acute fatal oral dosage for mammals is about 0.5 gram per kgm. of body weight and 0.08 to 0.15 gram per kgm. of body weight when injected intravenously or hypodermically.

Chronic intoxication. The known manifestations of chronic intoxication are: 1, mottled enamel, a hypoplasia of the enamel which is endemic in several areas in certain parts of the world; 2, osteosclerosis, a bone disease occurring among cryolite workers in Denmark, and from high fluoride containing areas in Argentina, India and North Africa; 3, a disease similar to osteomalacia, observed among herbivora in the vicinity of certain factories (aluminum, super-phosphate) in Europe; 4, darmous, a dental and osseous disease among herbivora in North Africa; 5, gáddur, a dental and bone disease occurring among herbivora in Iceland, after volcanic eruptions. The first detectable symptom of chronic fluoride intoxication is a hypoplasia of the teeth. The most reliable data available indicate that the consumption of water containing 1 p.p.m. of fluorine during the period of calcification of the teeth results in the production of mottled enamel in 10 per cent or more of children using such water. As the fluorine concentration in the water increases, the number of children affected increases and the mottling becomes more severe. On the other hand, the incidence of dental caries tends to decrease when such waters are used for culinary purposes.

Mottled enamel and high fluoride containing drinking waters have

been found in several areas in thirty states in the United States and in numerous other countries in the world; hence it is a world-wide problem.

While the disturbance of tooth structure is the most common symptom of fluoride intoxication, reports from Denmark, Africa, India, Argentina, the United States and elsewhere indicate that the bones of man and other species consuming large quantities of fluorides for several years may be damaged. The fluoride content of bones and teeth increases significantly under these conditions. These reports are based upon the observations of a relatively small number of cases, but certain characteristic changes occur in all of them. Pathological changes in other organs and tissues, e.g., kidney, thyroid, lymph nodes, have also been reported. The data are limited; therefore they should be accepted cautiously.

There appears to be a significant variation in the toxicity of the different fluorine compounds and in the sensitivity of various species to the same fluorine compound.

Scientific and feasible practical methods are now available for the removal of excessive concentrations of fluorides from drinking waters. The fluoride content of high fluorine containing fertilizers and solid foods can be decreased by heat treatment under favorable conditions.

FURTHER RESEARCH SUGGESTED

1. There is a need for more complete data regarding the occurrence of fluorides in public and private water supplies, foods, drugs, fertilizers and soils.

2. More complete surveys should be made to determine the number of people with mottled enamel, and its correlation with the concentration of fluorides in drinking waters.

3. Additional fluoride balance studies should be made on man and other species to determine the minimum concentrations of various fluorine compounds required to produce pathological changes in tooth, bone, kidney, and other vital structures of the body. Clinical and radiological studies should be made on man, particularly aged adults, living in endemic mottled enamel areas.

4. Further research on practical methods of removing excessive concentrations of fluorides from drinking water, food products, and fertilizers should be encouraged.

5. More long range experiments are needed to determine the effects (beneficial or toxic) of small quantities of fluorides in the diet of man and other species.

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